FY2003 Final Report

Ecological Risk Assessment of Perchlorate In Avian Species, Rodents, Amphibians and Fish

SERDP Project ER-1235

October 2005

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A FINAL REPORT

Reproductive and Molecular Toxicity of TNX in Deer Mice.

STUDY NUMBER: MRT-03-01

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RESEARCH INITIATION: October 2003

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GOOD LABORATORIES PRACTICES STATEMENT

This study was conducted in the spirit of the Good Laboratory Practice Standards whenever possible (40 CFR Part 160, August 17, 1989).

Submitted By:

_____ _____
George P. Cobb Date

_____ _____
Quality Assurance Manager Date

1.0 **DESCRIPTIVE STUDY TITLE**: **Reproductive and Molecular Toxicity of TNX in Deer Mice.**

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5.0 **PROPOSED EXPERIMENTAL START & TERMINATION DATES**:
Start Date: April 1, 2003
Termination Date: December 31, 2003

6.0 **KEY PERSONNEL**:
Dr. George P. Cobb, Study Director / Study Advisor
Mr. Jordan Smith, Research Assistant
Mr. Nick Romero, Student Assistant
Ms. Xiaoping Pan, Research Assistant
Dr. Ronald J. Kendall, Testing Facility Management

7.0 STUDY OBJECTIVES / PURPOSE:
- To evaluate the reproductive and genotoxic effects of TNX on rodents (Peromyscus maniculatis).

8.0 **TEST MATERIALS**:

Test Chemical name: hexahydro-1,3,5-trinitroso-1,3,5 triazine
CAS number: 13980-04-6
Characterization: 99% pure
Source: SRI International

9.0 JUSTIFICATION OF TEST SYSTEM:

Deer mice will be used in this study because they are a sentinel wildlife species. In addition, they are easy to handle and it is economical to maintain them. There are also available microsatellite primers to evaluate genotoxicity in these mice.

10.0 **TEST ANIMALS**:

Species: Peromyscus maniculatis
Strain: Laboratory
Age: voles (conception - >100 days)
Number: approximately: 300
Source: Peromyscus Stock Center, USC, Columbia, SC.

11.0 **PROCEDURE FOR IDENTIFYING THE TEST SYSTEM**:

All mice were placed in standard rodent cages and each cage was labeled with a note card containing the appropriate identification information for the animal. When housed communally, offspring were identified by ear clipping patterns. Collected samples were placed in individually labeled bags/containers and stored appropriately.

12.0 **EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL**:

There will be 6 pairs of animals per treatment and 4 treatment groups; 0 ppb, 0.1 ppb, 10 ppb, and 100 ppb TNX, This provides 48 adult animals plus the offspring from three litters from each of these pairs. TNX was selected as a toxicant since it is an N-Nitoros metabloite of RDX. Long term RDX exposure has produced adenoma and carcinoma in rodents.

13.0 **METHODS**:

Dosing

Dose Preparation

Hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX) (>99 % pure) was obtained from SRI International (Menlo Park, CA, USA). Dosing solutions were renewed every 3 d and analyzed with reverse phase high performance liquid chromatography to ensure concentration. Dosing water bottles were weighed at the time of water change to monitor water consumption by deer mice.

Animals

Twenty three breeding pairs of virgin male and female deer mice were obtained from the Peromyscus Genetic Stock Center (University of South Carolina, Columbia, SC, USA). Mice were allowed to acclimate for one week. Breeding pairs were housed separately in standard rodent cages (approximately 29 x 18 x 14 cm) lined with laboratory grade aspen bedding (Harlan Teklad, Madison, WI, USA). Cages were located in rooms with temperatures ranging from 18.3-25.6° C, 25-75% relative humidity, and 16:8 h light:dark period. Food and water were provided manually, checked daily, and available ad libitum to mice. Food used was Purina Certified Rodent Chow® No. 5002 (Purina Mills, St. Louis, MO, USA). Experiments and housing were conducted in compliance with Texas Tech University's Animal Use and Care Committee's guidelines.

Experimental procedure

Once accilmated, deer mice were randomly paired. Six pair were assigned to each exposure group (1 µg/L, 10 µg/L, and 100 µg/L), and five pair were assigned to the control group. Hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX) was administered ad libitum in drinking water. Deer mice bred and produced three litters of offspring (F1A-C). Offspring from F1A and F1B litters were weaned on post-natal day (PND) 21 and were dosed until sacrifice on PND 45. These offspring were weighed at weaning and again every seven days afterwards until sacrifice. Offspring in the F1C litter were weighed at PND 2, 5, 10, 15, and 20. These offspring were sacrificed on PND 21 using the same procedure as F1A and F1B mice.

Euthanasia was conducted using carbon dioxide gas as an asphyxiant. At necropsy, blood was obtained from all animals via heart puncture and used in biochemical analyses as part of an allied study. The following organs were removed: liver, kidney, gonads, and brain. Parent mice (F0) were sacrificed on the same day as their F1C offspring. Endpoints investigated on the offspring include: number born, survival, weight gain, organ weights, and liver TNX residue.

Residue Analysis

Liver samples were divided into two groups: one for TNX residue analysis, and one for cytochrome P450 2B analysis in an allied study (results not yet available). Livers within litters from common dose groups were composited to produce sufficient sample masses (~1g) for trace analyses. Liver samples were then homogenized and extracted using Accelerated Solvent Extraction (Dionex, Salt Lake City, UT, USA). The extract was then cleaned first using a Florisil solid phase extraction cartridge (Supelco, Bellefonate, PA, USA), then with a styrene-divinylbenzene cartridge (Supelco, Bellefonate, PA, USA). Gas chromatography was conducted

using an electron capture detector (Agilent, Palo Alto, CA, USA) and a 30-m x 0.25-mm-i.d x 0.25 µm film thickness HP-5 column (Hewlett-Packard Company, Wilmington, DE, USA). The temperature profile initiated at 90° C, maintained for 2 min, ramped to 130° C at a rate of 25° C/min, ramped to 200° C at a rate of 10° C/min, and finally, ramped to 250° C at a rate of 25° C/min. This method allows recovery of 88.1 ± 13.2 % (relative standard deviation) with a detection limit of approximately 20 µg/L [26]. Liquid chromatography-mass spectroscopy in negative ion mode was used to verify TNX presence and concentration. Acetic acid was added to create a TNX-acetic acid complex (233 amu). A primary peak of 233 amu was monitored in the analysis.

Statistics

Litter size, body weights, relative organ weights and absolute organ weights were evaluated using a one-way analysis of variance coupled with the Tukey-Kramer test [27]. Offspring mortality was evaluated using a chi square test of independence for overall and developmental period mortality. Developmental periods evaluated included birth (live versus stillbirths), birth to PND four, PND four to weaning (PND 21), and weaning to sacrifice (PND 45). All statistical tests used an α value of 0.05. R Version 2.0.0 [28] was the software used to analyze data.

Molecular Toxicology

Determination of EROD activity

Three litters of deer mice (F1A, F1B and F1C) were initially dosed with 1ppb, 10ppb, and 100ppb of TNX (Hexahydro-1,3,5-trinitroso-1,3,5-triazine), an RDX metabolite. F1A and F1B groups were weaned and dosed till post natal day 45 day and then sacrificed, with the exception of the F1C group, which were sacrificed at weaning where brain, liver, plasma, kidney and gonads were isolated and stored at -80°C.

Microsomal Preparation

Liver microsomes were prepared according to SOP BC-3-01-01. Whole livers were weighed and recorded then homogenized with 1ml ice-cold buffer (pH 7.4) containing 250mM sucrose and 20mM Tris, using a teflon pestel. The homogenates were centrifuged at 10,000 x g for 10 minutes at 4°C, and the supernatant obtained was centrifuged at 105,000 x g for 70 minutes at 4°C. The supernatant (the cytosol fraction) was discarded. A wash buffer with 25mM KCl was used to rinse the pellet to remove any lipid residue that may have collected on it. The pellet was homogenized in 1ml of a separate resuspension buffer containing 20% (volume) glycerol. Protein concentrations were measured using the Bradford assay with bovine serum albumin (BSA) (Sigma) as the protein standard and the resultant microsomal fraction was stored in 1ml aliquots at -80°C.

Monooxygenase Activity Assay

Ethosyresorufin O-Deethylase (EROD) activities were measured using SOP BC-2-10-01 with modifications. The assays were optimized and carried out on clear 96-well plates (Corning Costar) for deer mouse. Incubations were performed in a solution consisting of 140µl of EDTA and 0.1M Tris Buffer (.0014mM), 10µl ethoxyresorufin (5µM in acetone), and 20µl of microsome preparations per well. Assay plates were pre-incubated for 30 min at 37° C prior to

addition of 10µl NADPH (.005M). Kinetics of appearance of resorufin (λex=544nm, λem=590) from ethoxyresorufin was followed using SoftMax Pro Software.

One-dimensional Polyacrylamide Gel Electrophoresis and Western Blot Analyses

Tissue processing. Deer mice liver samples were removed from the -80 C freezer and approximately 100 mg of tissue was homogenized in 1ml of TRIS-sucrose-EDTA buffer (7.5 g Tris base, sucrose, ethylenediamine tetra-acetic acid). Protein concentration of the homogenates were determined from a standard curve, using bovine serum albumin (BSA) as the standard, and Bio-Rad's protein determination assay.

Proteins from each homogenate were separated according to the method of Laemmli (1970). Briefly, samples were heated 100 C for 5 min in SDS-PAGE solubilization buffer (10% glycerol, 5%*β*-mercaptoethanol, 3% SDS, 0.01% bromophenol blue in 62.5 mM Tris (pH 6.8). The samples were loaded on a 4-15% gradient gel (Bio-Rad) and separated using a Bio-Rad Mini-gel electrophoretic apparatus.

Immunologic detection of Monooxygnease proteins. Following electrophoretic separation, the gel, filter papers, and nitrocellulose paper were saturated with transfer buffer (25 mM Tris, 192mM glycine, 20% methanol, adjusted to pH 8.3). Electrophoretic "blotting" of proteins onto nitrocellulose paper was accomplished using 4.0mA constant current overnight at 4°C. The nitrocellulose blot was placed in Tris-buffered blocking solution (TBS; 20 mM Tris, 500 mM NaCl, pH 7.5) containing 4% nonfat dry milk with gentle agitation for 4 hours at room temperature. After blocking, the blots were incubated for 21 hours with monoclonal antibody derived against specific proteins, namely cytochrome P4501A1 and 2B1 in the liver. Blots were detected by chemiluminescence.

Following incubation with the primary antibody, the blot was washed in 6 changes of TTBS containing 5% nonfat dry milk with vigorous shaking for 45 minutes at room temperature. The blot was then incubated with horseradish peroxidase-conjugated goat antimouse secondary antibody (Tris base, 0.1% tween-20, 5% milk) with gentle agitation for one hour at room temperature. Following incubation, the blot was washed in 2 changes (for 15 minutes at each change), of TBS with 0.05% tween with vigorous shaking over 45 minutes. The blots were rinsed twice in TBS. Blots were visualized using enhanced chemiluminescence's detection development according to manufacturers specifications (Amersham). Autoradiographs were developed and analyzed utilizing ChemilImager software.

Genotoxicity

Ribonucleic acid (RNA) isolation

Total RNA was extracted from deer mice tissues using TRIzol Reagent (Invitrogen, Carlsbad, CA). The working area was made RNase free using RNaseZAP (Ambion, Austin, TX). Care was taken not to cross-contaminate between the tissue samples. Tissue (50-100mg), while still frozen, was homogenized in 1ml of TRIzol and RNA was isolated following the manufacturer procedure

RNA purity and integrity
The ratio of the absorbance at 260nm and 280nm was observed and used to assess the purity of RNA. An A_{260}/A_{280} ratio of 1.8 to 2.1 is indicative of highly purified RNA.

Integrity of the isolated RNA was checked to make sure that only high quality RNA is used in gene expression analysis. Total RNA integrity was assessed by running the RNA sample on a 1% formaldehyde agarose gel with ethidium bromide. Intact total RNA on such a denaturing gel will have sharp, clear 28S and 18S ribosomal RNA (rRNA) bands. Gel preparations with intact and degraded RNA samples showed easily observable differences (Figure 1). A sample solution containing 1µg RNA sample, 8-10µl of formaldehyde loading buffer, and 0.3 µl ethidium bromide was prepared for each sample and incubated at 75°C for 15 minutes. An aliquot of 10µl of each sample solution was run on a 1% denaturing formaldehyde agarose gel along with an 18S + 28S Ribosomal RNA from calf liver (Sigma-Aldrich, St. Louis, MO), which was used as the molecular weight marker.

Electrophoresis was performed at 80 volts for 2 hours in1X MOPS-EDTA-Sodium Acetate (MESA) buffer. ChemiImager 4400 (Alpha Innotech Corporation, San Leandro, CA) was used to take the photograph of the gel under ultra-violet (UV) light. Only the samples for which two clear bands were resolved were used in downstream applications.

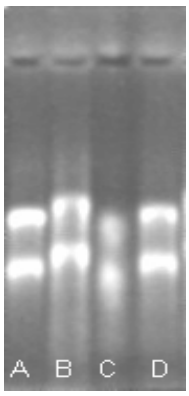

Figure 1: RNA Integrity Gel. (A) 18S + 28S Ribosomal RNA Standard from calf liver, (B) Partially denatured RNA sample, (C) Completely denatured RNA sample, (D) Intact RNA sample.

Reverse transcription
A 2µg quantity of each RNA sample was reverse transcribed using RETROscript First Strand Synthesis Kit (Ambion, Austin, TX) to generate deer mice complementary DNA (cDNA) for amplification and gene isolation. An aliquot of 2µl of Oligo(dT) was added to the sample and nuclease free water was added to a final volume of 12µl. Heat denaturation of RNA was done at 75°C for 3 minutes. An aliquot of 2µl of 10X RT buffer, 4µl of dNTP mix, 1µl of RNase Inhibitor and 1µl of Moloney Murine Leukemia Virus-Reverse Transcriptase (MMLV-RT) were added and the samples were incubated at 43°C for one hour for reverse transcription. MMLV-RT has significantly less RNase activity and was used in this study, as the aim was to amplify full-length cDNA molecules (Bustin 2000). Finally, the samples were incubated at 92°C for 10 minutes to inactivate the Reverse Transcriptase. The prepared cDNA samples were stored in -20°C.

Primer and probe design
The deer mice specific sequence was submitted to Primer Express software (Perkin-Elmer Applied Biosystems, Foster City, CA) to design the TaqMan specific probe and oligonucleotide primers for real time PCR amplification of AhR mRNA equivalents. The TaqMan probe and primers for deer mice AhR gene designed in our lab and synthesized by Applied Biosystems. FAM (6-carboxyfluorescein) was used as the 5' reporter dye and TAMRA (6-carboxytetramethylrhodamine) was used as the 3' quencher dye. The internal standard, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers and probes were designed in our lab and synthesized by Applied Biosystems with a VIC (Applied Biosystems, Foster City, CA) 5' reporter and a TAMRA 3' quencher.

Quantitative real-time PCR
Quantitative real-time PCR analysis by standard curve quantitation was done using ABI PRISM 7000 Sequence Detection System (Perkin-Elmer Applied Biosystems, Foster City, CA). GAPDH mRNA was used as the internal housekeeping control in each well to normalize the target signal. GAPDH is one of the most widely used internal housekeeping controls and the RNA encoding GAPDH is ubiquitous and truly invariant (Winer et al., 1999). rRNA controls were not used for normalization as the quantitating targets had been enriched for mRNA.

Amplifications were conducted in a final volume of 50 µl containing 250nM TaqMan GAPDH probe labeled with the 5' reporter dye VIC, 20nM forward and reverse GAPDH primers, 250nM TaqMan AhR probe labeled with the 5' reporter dye FAM, 10µM forward and reverse AhR primers, 50ng of cDNA, and 25µl of TaqMan Universal PCR master mix containing ROX dye as a passive reference (Perkin-Elmer Applied Biosystems, Foster City, CA). TAMRA was used as the quencher dye in both the probes. Four 10-fold dilutions of pooled control cDNA samples were used as the standards. Three reactions with no cDNA template were used as the No Template Controls (NTC). The C_T values for the FAM and VIC dyes of the standards were used to generate the FAM and VIC standard curves respectively. The C_T values from the FAM and VIC dyes of each unknown sample were used to extrapolate the amount of their mRNA equivalents from the FAM and VIC standard curves respectively (Young et al., 2002). The ratio of the mRNA equivalents of AhR (FAM dye) and GAPDH (VIC dye) was then taken as the value of AhR gene expression relative to GAPDH.

14.0 RESULTS

Survival

Overall mortality rate of offspring increased in all dose groups for all litters when compared to the control. When using a chi square analysis ($\alpha = 0.05$) to evaluate statistical significance, the specific differences were found in F1A overall mortality (χ^2~11.4, P~0.001-0.01), F1A birth to post natal day 4 mortality (χ^2~8.5, P~.02-.05), F1B overall mortality (χ^2~11.0, P~0.01-0.02), and F1B birth mortality (χ^2~17.3, P~0.0001-0.001).

Results show a dose response in body weight to TNX during early growth of F1C mice-birth to PND 21 (Figure 2). The 10 µg/L F1B mice show significantly lower body weight from all other dose groups at PND 21, as well as, differences from1 µg/L and 100 µg/L dose groups at PND 28 (Figure 3). F1A mice show no significant differences in body weights from PND 21-PND 45 (Figure4).

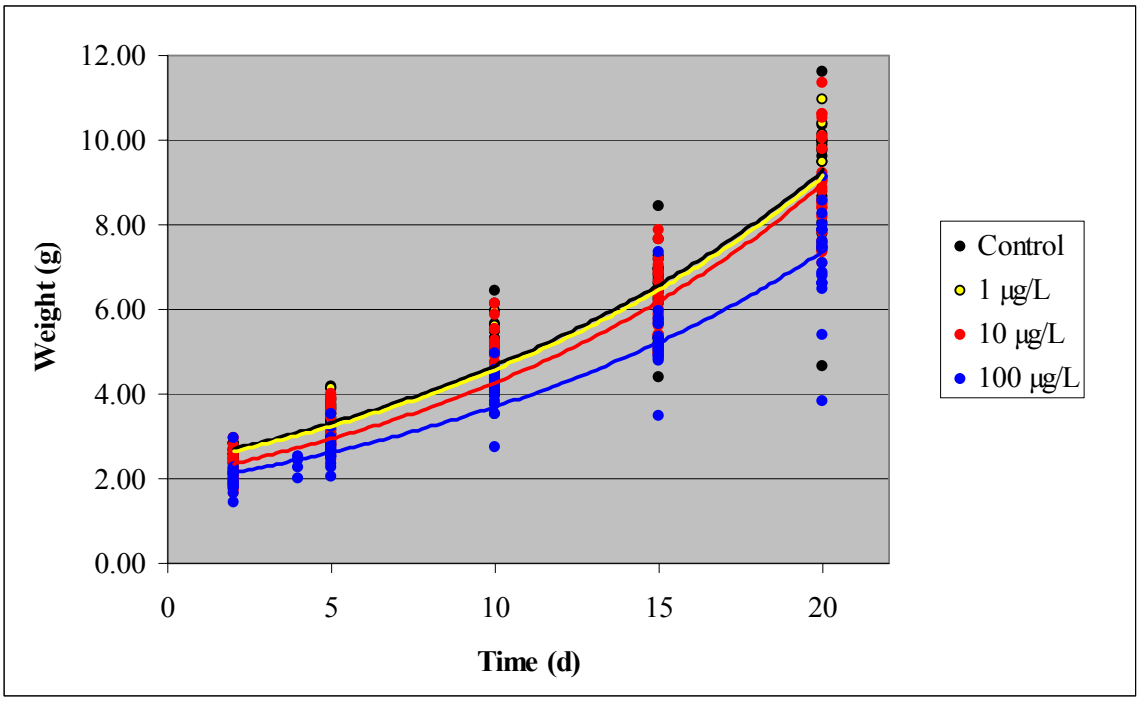

Figure 2. Growth Curve of F1C Deer Mice Offspring Dosed with Varying Concentrations of TNX in Drinking Water.

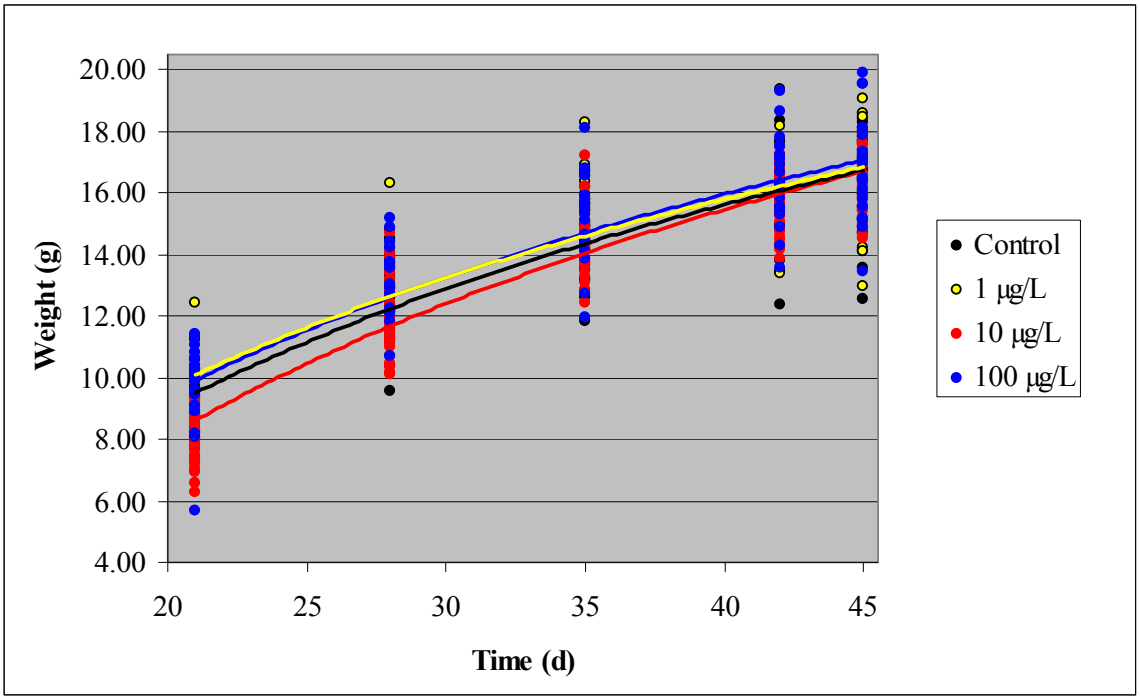

Figure 3. Growth Curve of F1B Deer Mice Offspring Dosed with Varying Concentrations of TNX in Drinking Water.

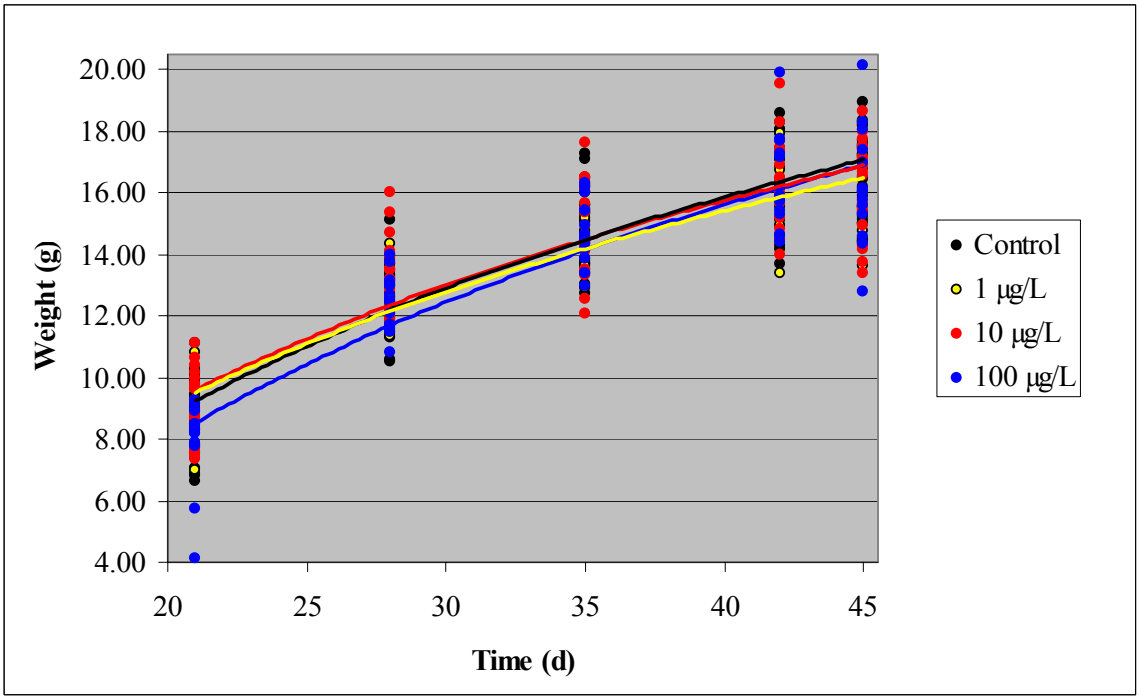

Figure 4. Growth Curve of F1A Deer Mice Offspring Dosed with Varying Concentrations of TNX in Drinking Water.

Relative organ weights of brain, kidneys, testes, and liver in F1A and F1B litters showed no significant differences (Table 1). F1C 100 µg/L mice showed a significant increase in relative brain weight when compared to all other dose groups (P~0.0006-0.304). However, absolute brain weights for the same mice showed no significant differences (P~0.96). Absolute kidney weights of 100 µg/L F1C mice were significantly lower than all other dose groups (P~0.0000-0.0007). However, relative kidney weights showed no significant differences among the same mice (P~0.54). All other F1C relative organ weights showed no significant differences.

Table 1. Means with Standard Deviations of Absolute Organ Weights from Deer Mice Offspring Dosed with TNX. Superscripts Represent Statistical Groupings.

Litter	Dose	Liver (g)	Kidney (g)	Brain (g)	Testes (g)
F1A	Control	0.703 ± 0.074^{a}	0.200 ± 0.020^{b}	0.412 ± 0.035^{c}	0.169 ± 0.020^{e}
	1 µg/L	0.696 ± 0.108^{a}	0.194 ± 0.020^{b}	0.413 ± 0.041^{cd}	0.164 ± 0.027^{e}
	10 µg/L	0.686 ± 0.118^{a}	0.197 ± 0.019^{b}	0.412 ± 0.045^{cd}	0.168 ± 0.022^{e}
	100 µg/L	0.671 ± 0.129^{a}	0.194 ± 0.019^{b}	0.396 ± 0.052^{d}	0.159 ± 0.015^{e}
F1B	Control	0.687 ± 0.103^{f}	0.208 ± 0.028^{g}	0.446 ± 0.058^{h}	0.174 ± 0.013^{i}
	1 µg/L	0.651 ± 0.144^{f}	0.208 ± 0.038^{g}	0.447 ± 0.074^{h}	0.180 ± 0.030^{i}
	10 µg/L	0.693 ± 0.084^{f}	0.199 ± 0.018^{g}	0.443 ± 0.054^{h}	0.161 ± 0.019^{i}
	100 µg/L	0.708 ± 0.099^{f}	0.213 ± 0.029^{g}	0.444 ± 0.053^{h}	0.179 ± 0.025^{i}
F1C	Control	0.349 ± 0.083^{jk}	0.143 ± 0.023^{l}	0.401 ± 0.041^{n}	0.0196 ± 0.006^{o}
	1 µg/L	0.405 ± 0.058^{j}	0.146 ± 0.020^{l}	0.405 ± 0.061^{n}	0.0228 ± 0.010^{o}
	10 µg/L	0.366 ± 0.058^{jk}	0.137 ± 0.014^{l}	0.404 ± 0.043^{n}	0.0185 ± 0.006^{o}
	100 µg/L	0.324 ± 0.061^{k}	0.117 ± 0.020^{m}	0.397 ± 0.048^{n}	0.0161 ± 0.004^{o}

Residue analyses

Liver TNX residue analysis showed little to no accumulation in control (all non-detects) and low dose groups (non-detect to 28 µg/g), while the medium and high dose groups showed more accumulation (20 to138 µg/g and non-detect to 140 µg/g respectively (Table 2). This suggests a dose response for TNX accumulation by mice. While the responses were variable, the mean TNX accumulation in the 100 µg/L dose group trended higher in sequential litters.

As discussed in the report of Analytical Core activities, we have validated a method for HE determination in blood samples. This method was used to determine that TNX was present in mouse blood from the higher two dose groups (Figure 5).

Table 2. Means with Standard Deviations of Liver TNX Residues of Deer Mice Offspring Dosed with TNX.

F1A	Concentration (ng/g)	No.	No. of Detects
Control	Below Detection	4	0
1 µg/L	Below Detection	2	0
10 µg/L	49.03 ± 20.62	2	2
100 µg/L	53.30 ± 14.90	2	2
F1B			
Control	Below Detection	4	0
1 µg/L	Below Detection	2	1
10 µg/L	93.21 ± 32.10	9	9
100 µg/L	87.93 ± 68.72	3	2
F1C			
Control	Below Detection	3	0
1 µg/L	Below Detection	2	0
10 µg/L	54.955 ± 14.67	3	3
100 µg/L	121.78 ± 49.76	4	4

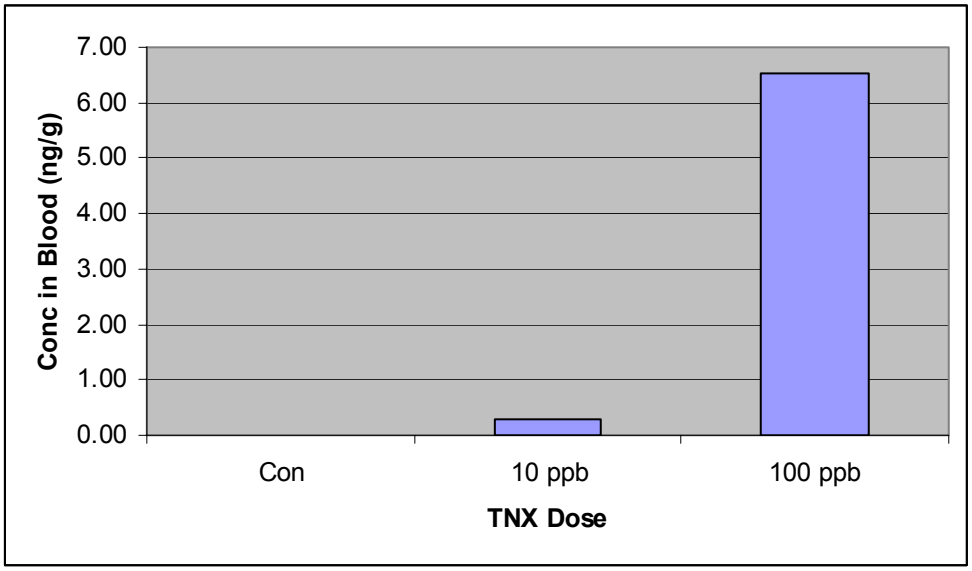

Figure 5. TNX accumulation in mouse blood following aqueous dosing.

MOLECULAR BIOLOGY

Molecular Toxicology Results and Discussion

A large number of chemicals induce EROD activity in a variety of species. Studies on mechanisms of CYP1A1 -induced toxicity suggests that EROD activity may not only indicate chemical exposure, but also may precede effects at various levels of biological organization (Whyte et. al., 2000). This study was carried out to determine the effect of TNX on the activity and expression of EROD. An analysis of the data for homogeneity of variation indicated that the

data was not homoscedastic. The data was therefore log transformed and analysis of variance done using Minitab software. The results of this study indicate that TNX did not affect the protein expression or the activity of EROD as measured by western blot and enzyme activity, respectively. Western blotting showed no significant difference among the treatment groups in expression of CYP 1A1 and 2B1. The results are shown in Figures 6-8.

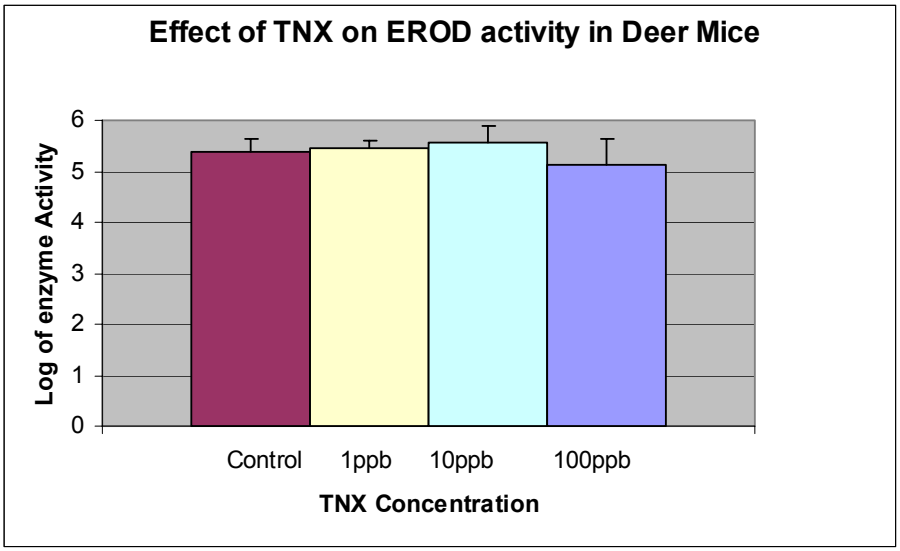

Figure 6 The effect of TNX on EROD activity of Deer Mice .

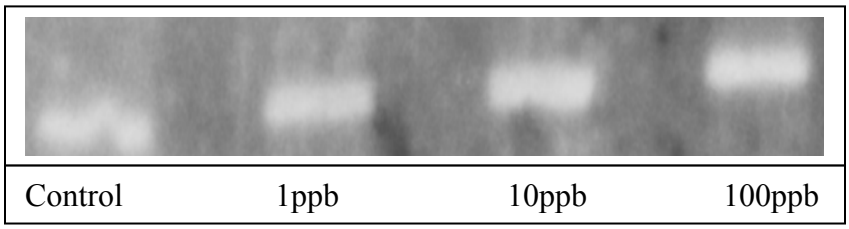

Figure 7 showing the effect of TNX on CYP1A expression.

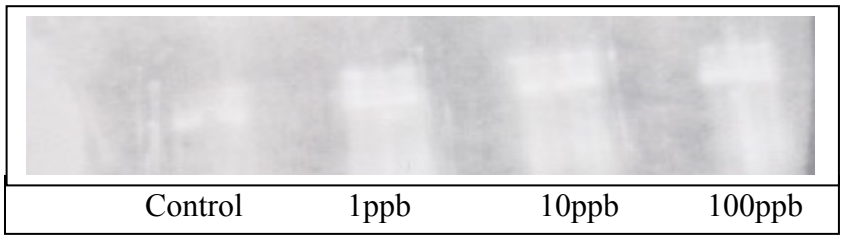

Figure 8 showing the effect of TNX on CYP2B expression

Genotoxicity

DNA microsatellite mutation rate was determined by isolating DNA from liver tissue using PureGene® DNA Extraction Kit and amplifiying fifteen microsatellite loci from each subject for DNA analysis using a thermocycler. Actual mutations were determined with an ABI Prism-3100 Gene analyzer. We evaluated DNA from a total of 375 mice and found mutation frequencies in the range observed for rodents, 0.001 to 0.0001 (Dallas, 1992). There was no significant difference in the numbers of mutations between dose groups (Chi Sq=0.24). Some individuals were omitted from the data analysis due to questionable microsatellite masses. Once these microsatellites are revisited there is a slight possibility that significant a difference could be seen.

Table 3. Mutations across 15 DNA microsatellites of mice exposed to increasing concentrations of aqueous TNX.

Dose Group	Mutation Frequency
Control	**6.51E-03**
1 µg/L	**8.61E-03**
10 µg/L	**4.88E-03**
100 µg/L	**4.79E-03**

Gene expression

We have successfully developed deer mice specific AhR probe and primers. Furthermore, we have established the expression of the AhR in several deer mice tissue. The Aryl hydrocarbon receptor is a ligand-activated transcription factor that is associated with gene regulation. One set of genes associated with AhR regulation, are cytochrome P450 1A and 1B forms. The AhR binds aryl hydrocarbons forming complex that enter the nucleus of cells where biotransformation processes for the excretion of hydrocarbon compounds are activated. Furthermore, interaction with the Ah-receptor may result in the biosynthesis of new proteins with regulatory properties in cell growth and differentiation. For this reason such chemicals are potential teratogens and may induce carcinogenic consequences. We have utilized real time PCR to determine the expression level of AhR in deer mice tissue. The expression of the AhR in deer mice tissue relative to GAPDH is displayed in Figure 10. Surprisingly, the data indicate that the level of expression in the testes is several fold higher that any other tissue. Based on the rapidly dividing cells of the testes, and the potential susceptibility of this organ to toxic agents it appears that the expression of the AhR at such levels provided one means of protection for rapidly dividing spermatogenic cells. Furthermore, the current data provide promising directions for future research on the comparative biochemistry and molecular biology of the AHR in the deer mice following exposure to environmental contaminants.

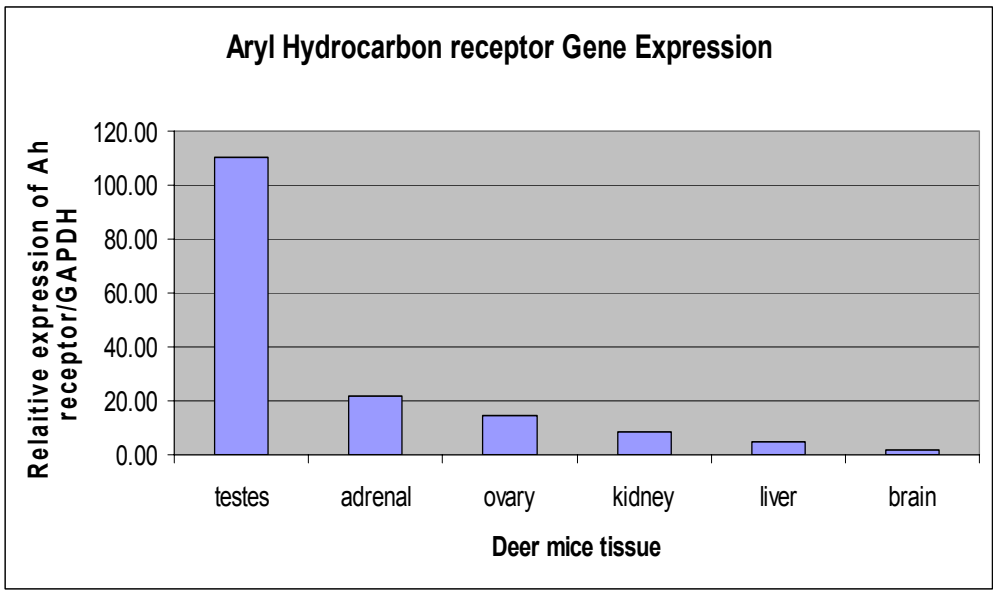

Figure 9. Relative expression of the Aryl Hydrocarbon receptor gene expression in Deer mouse tissue.

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TITLE: Developmental Response of Larval *Xenopus laevis* to TNX

STUDY NUMBER:	TNX-03-01
SPONSOR:	Strategic Environmental and Research Development Program SERDP Program Office 901 North Stuart Street, Suite Arlington, VA 22203
CONTRACT ADMINISTRATOR:	The Institute of Environmental and Human Health Texas Tech University/TTU Health Sciences Center Box 41163 Lubbock, TX 79409-1163
TESTING FACILITY:	The Institute of Environmental and Human Health Texas Tech University Box 41163 Lubbock, TX 79409-1163
TEST SITE:	The Institute of Environmental and Human Health Texas Tech University Box 41163 Lubbock, TX 79409-1163
RESEARCH INITIATION:	April 28, 2005
RESEARCH COMPLETION:	December 31, 2005

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GOOD LABORATORIES PRACTICES STATEMENT

This study was conducted in accordance with established Quality Assurance Program guidelines and in the spirit of Good Laboratory Practice Standards whenever possible (40 CFR Part 160, August 17, 1989).

Submitted By:

_____ _____

Ernest E. Smith, Ph.D Date

QUALITY ASSURANCE STATEMENT

This study was conducted under The Institute of Environmental and Human Health Quality Assurance Program and whenever possible to meet the spirit of the Good Laboratory Practices as outlined in 40 CFR Part 160, August 17, 1989.

Submitted By:

_____ _____

Ryan Bounds
Quality Assurance Manager

Date

1.0 DESCRIPTIVE STUDY TITLE:
Developmental Response of *Xenopus laevis* to TNX

2.0 STUDY NUMBER:
TNX-03-01

3.0 SPONSOR:
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SERDP Program Office
901 North Stuart Street, Suite
Arlington, VA 22203

4.0 TESTING FACILITY NAME AND ADDRESS:
The Institute of Environmental and Human Health
Texas Tech University
Texas Tech University Health Sciences Center
Box 41163
Lubbock, TX 79409-1163

5.0 PROPOSED EXPERIMENTAL START AND TERMINATION DATES:
Start Date: April 28, 2005
Termination Date: December 31, 2005

6.0 KEY PERSONNEL:
Ernest E. Smith, Co-Principal Investigator
Angella Gentles, Study Director
Ryan Bounds, Quality Assurance Manager
Ron Kendall, Principal Investigator and Testing Facility Management

7.0 STUDY OBJECTIVES / PURPOSE:
To assess gonadal development in *Xenopus laevis* in response to TNX exposure by evaluating histological end points of selected organs.

8.0 STUDY SUMMARY:
In this study, *Xenopus laevis* larvae were exposed to TNX from stages (1-10) for 70 days post fertilization. On day 70 the tadpoles were euthanized in bicarbonate buffered MS-222. Subsequently they were staged, measured, weighed, and sectioned for gonadal histopathology.

9.0 TEST MATERIALS:
Test Chemical: TNX
CAS: 13980-04-6
Source: TIEHH
Characterization: explodes when heated
Test Medium: Deionized Water

10.0 JUSTIFICATION OF TEST SYSTEM:

Although the toxicity of high explosive compounds such as RDX and HMX in fish and aquatic invertebrates has received some attention (Stevens et al., 2002; Lotufo et al., 2001; Hovatter et al., 1997; Burton et al., 1994; Peters et al., 1991), toxicity to other aquatic organisms, such as amphibian larvae, has not been determined. Similar to the situation in fish, information on the toxicity of metabolites of high explosives is also lacking in amphibians. Amphibians are currently a major focus of research because of worldwide population declines and occurrence of deformities (Pechmann et al., 1991; Schmidt, 1997; Kavlock, 1998). Recent evidence has also suggested that exposure to contaminants increases amphibian's susceptibility to effects of other environmental agents (Burkhart and Cline, 1999). Furthermore, amphibians are particularly sensitive to contaminant-induced effects such as embryo-larval developmental abnormalities and sex steroid endocrine disruption (ASTM, 1998; Hayes, 2000). Such effects have the potential to affect amphibian reproduction, and ultimately, growth and sustainability of amphibian populations. Thus, the aim of this subproject is to determine the developmental and reproductive toxicity of RDX, HMX, and RDX metabolites (TNX and MNX) in amphibians. Toxicity endpoints will include induction of embryo-larval developmental abnormalities, sex ratios, rate of metamorphosis, and gonadal histopathologic and steroidal changes. This study is of vital importance because little toxicological information exists on high explosives and their metabolites in general and specifically on the reproductive system of amphibians. MNX and TNX are metabolites of RDX, a compound that is widely used as an explosive both commercially and by the military. Large scale manufacture, use, and improper disposal of RDX are suggested to be problems which have led to severe contamination of soil and ground water by this compound and its metabolites. The proposed studies will aid in predicting the impact of cyclic nitroamines and their metabolites on a common laboratory amphibian model species-*Xenopus laevis*. The proposed focus of this study is to expand our knowledge of HMX, RDX, and RDX-metabolite contamination using laboratory studies designed to characterize and quantify ecological risks associated with exposure to these compounds in the ecosystem. This research project will address data gaps related to RDX-metabolites on amphibians and will provide data for ecological risk assessment, as we have successfully accomplished with our perchlorate program.

11.0 TEST ANIMALS:

Species:	*Xenopus laevis*
Strain:	Wild type
Age:	larvae
Sex:	Males and females
Number:	400
Source:	In house breeding colony.

12.0 PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:

Individual identification of the animals was not applicable, however, each beaker was labeled as indicated in TIEHH SOP **AQ-1-17,** which includes genus and species name; common name; project name, number, and start date; and the name of the person responsible for animal care.

13.0 EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:

The study consisted of 4 treatment groups, namely, control, 250ppm, 500ppm and 1000ppm TNX. There were five replicates for each treatment group and each replicate had 20 animals.

14.0 METHODS:

14.1 Test System acquisition, quarantine, and acclimation.

The larvae were obtained by breeding animals that are reared at the institute. Therefore, no quarantine was necessary.

14.2 Assignment of Animals to Study Group and Identification

The eggs/larvae collected from three females were combined in one aquarium containing FETAX, and the larvae were randomly selected and placed in each beaker containing FETAX.

1.

14.3 Test Material Application

Rates/concentrations: *Xenopus laevis* larvae were exposed to TNX at concentrations of 0, 250, 500, and 1000 ppm.

Frequency: Application frequency was continuous as the test substance was supplied constantly in the FETAX medium.

Route/Method of Application: The animals were exposed orally, dermally, and via the lungs.

14.4 Daily Observations

Animals were monitored daily for changes in general health. Any changes were noted on the appropriate forms.

14.5 Animal Euthanasia and Sample Collections

After 70 days of exposure the tadpoles from each tank were removed using a fish net. They were placed in a beaker containing bicarbonate buffered MS-222 for euthanasia. They were weighed, measured and staged and then placed in Bouin's solution for 48hrs, followed by a transfer to 70% alcohol.

14.6 Evaluations

Evaluations in this report were reduced to percent hatch and mortality rate, snout-vent length, weight, and stage of development. Significant amount of effort was put into evaluating the mortality problem and measures for that would be required to eliminate the high larval mortality rate that were present over the period of the study. This study was repeated three times over the course of the study period (for a total of 4 trials) due to unacceptable high larval mortality. In each case both the test animals and controls were observed with an unacceptable level of mortality that was greater than 45% in some of the repeated evaluations. Due to the unacceptable high mortality rate among the groups including the control animals no statistical evaluation was carried out on the raw data collected during these exposures. Histology was also done to assess the gonads.

15.0 RESULTS and DISCUSSION:

The results presented in this report represent data for 4 consecutive trials of the experiment. Due to a persistent problem with high mortality rate (Table 1) even among the control groups, the data was not statistically analyzed. No valid scientific deduction or conclusion concerning the toxicity of TNX can be drawn from these results. As can be seen from the results in Table 2, there was an unidentified problem with the overall development of the tadpoles. Based on previous studies in our lab, using the same standard operating protocol, the developmental stage of the tadpoles were lower than expected after post-hatch day 70. In addition, the growths of these tadpoles were obviously retarded and thus they were much smaller than expected. For the reason, indicated above, the report and discussion will focus on the solution to the problem faced with the experiment and the measures taken to prevent this from reoccurring.

Trial	Termination day	Mortality in control
1	70	89.0%
2	35	38.0%
3	70	94.0%
4	70	63.0%

Table 1 showing mortality rate among control animals in the 4 trials.

As indicated above, this study was attempted 4 times. Three trials were taken to day 70, the duration indicated in the protocol; the second trial was terminated prematurely on day 35 due to the unacceptable level of larval mortality.

TNX concentration	% hatch	Average mortality	average stage
0	87.00	63.422	49.56
250	93.00	64.839	52.20
500	86.00	70.147	51.58
1000	90.00	54.268	53.46

Table 2 showing hatch rate, mortality rate and average stage of tadpoles at post-hatch day -70 in trial 4.

Several initiatives were taken to improve the survival rate of the tadpoles. Though these steps improved the survival rate, the number of animals that died by the end of the 70 day exposure rendered the data collected useless still. The initiatives that were taken included treating all the breeders with antibiotics for a week (both gram positive and negative) then treating them for 2 weeks with a copper sulfate medication. The frogs were given time to excrete the antibiotics and or their metabolite as well as the copper sulfate. A second water purification system and a UV filter were installed to sterilize and improve the quality of the water used to prepare the media. The figure below is an illustration of the second system that was installed in-line with the primary treatment systems to purify the water in the lab. This would eliminate any source of contamination or microbe that could have by-passed the first treatment system. Numbers 1 and 2 represent the second UV and the filter systems, respectively.

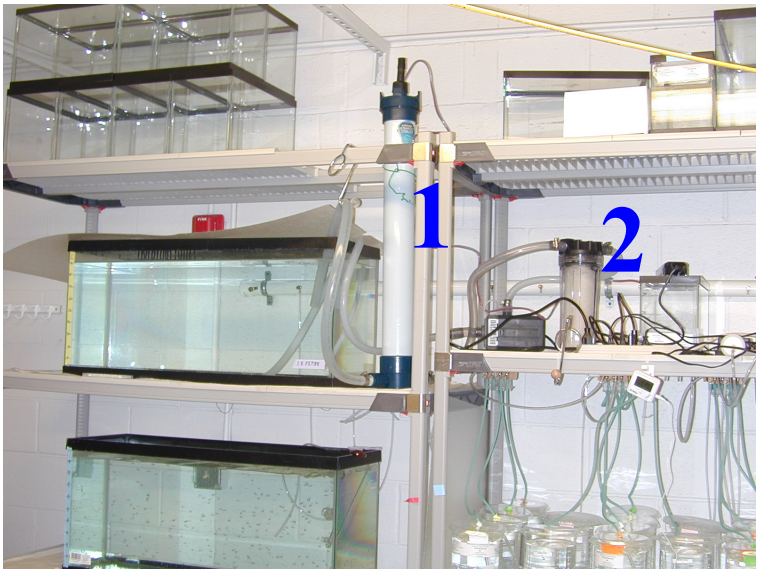

Figure 1 showing the purification system installed.

Subsequent to the additional treatment system and pre-experimental antibiotic treatment, we carried out a preliminary feeding study consisting 9 treatment groups. This study is currently underway to determine if feeding regimen and the brand of feed may play a role in the problem that we had. The design is as shown in the table below.

Group No.	Feed Brand	Number of times fed per day	Survival
I	German	1 time per day	18
II	German	2 times per day	18
III	German	1 time per 2 days	15
IV	Nasco	1 time per day	17
V	Nasco	2 times per day	12
VI	Nasco	1 time per 2 days	20
VII	Express	1 time per day	0
VIII	Express	2 times per day	0
IX	Express	1 time per 2 days	0

The survival rate in this preliminary study exceeds that in the TNX study, even among the groups that have similar feeding regimen (1 time per 2 days) as those in the TNX study. A major difference among these groups is that the tadpoles that are fed more frequently are much larger than those of others groups and are several folds bigger in comparison to those of the TNX study. A number of tadpoles in groups III and V died because they were aspirated during water change. Most noticeable is the fact that the express food groups were observed with 100% mortality. The data generated from this study will be provided to the sponsor as an addendum in upcoming reporting cycles.

16.0 STUDY RECORDS AND ARCHIVE:

Study records will be maintained at The Institute of Environmental and Human Health Archive for a minimum of one year from study completion date.

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17.0 REFERENCES

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TITLE: Effects of RDX and HMX on Larvae of *Xenopus laevis*

STUDY NUMBER:	RDX-03-01
SPONSOR:	Strategic Environmental and Research Development Program SERDP Program Office 901 North Stuart Street, Suite 303 Arlington, VA 22203
CONTRACT ADMINISTRATOR:	The Institute of Environmental and Human Health Texas Tech University/TTU Health Science Center Box 41163 Lubbock, TX 79409
TESTING FACILITY:	The Institute of Environmental and Human Health Texas Tech University/TTU Health Science Center Box 41163 Lubbock, TX 79409
TEST SITE:	The Institute of Environmental and Human Health Texas Tech University/TTU Health Science Center Box 41163 Lubbock, TX 79409
RESEARCH INITIATION:	September 12, 2003
RESEARCH COMPLETION:	December 31, 2004

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GOOD LABOARATORIES PRACTICES STATEMENT

This study was conducted in accordance with established Quality Assurance Program guidelines and in the spirit of Good Laboratory Practice Standards whenever possible (40 CFR Part 160, August 17, 1989).

Submitted By:

_____ _____

Dr. Ronald J. Kendall Date
Principle Investigator / Testing Facility Management

QUALITY ASSURANCE STATEMENT

This study was conducted under the Institute of Environmental and Human Health's Quality Assurance Program and whenever possible to meet the spirit of the Good Laboratory Practices as outlined in 40 CFR Part 160, August 19, 1989.

Submitted By:

_____ _____

Ryan Bounds
Quality Assurance Manager

Date

1.0 DESCRIPTIVE STUDY TITLE:
Effects of RDX and HMX on Larvae of *Xenopus laevis*

2.0 STUDY NUMBER:
RDX-03-01

3.0 SPONSOR:
Strategic Environmental and Research Development Program
SERDP Program Office
901 North Stuart Street, Suite 303
Arlington, VA 22203

4.0 TESTING FACILITY NAME AND ADDRESS:
The Institute of Environmental and Human Health
Texas Tech University / Texas Tech University Health Sciences Center
Box 41163
Lubbock, Texas 79409

5.0 PROPOSED EXPERIMENTAL START & TERMINATION DATES:
Start Date: September 12, 2003
Termination Date: December 31, 2004

6.0 KEY PERSONNEL:
Christopher Theodorakis, Study Director
Ronald Kendall, Testing Facility Management
Todd Anderson, Assistant Director for Science
Ryan Bounds, Quality Assurance Manager

7.0 STUDY OBJECTIVES / PURPOSE:
To determine the acute toxicity and effects of the RDX and HMX on growth and development.

8.0 STUDY SUMMARY:
Xenopus larvae were exposed to seven concentrations of RDX or HMX, and TNX in separate experiments. They were exposed to these contaminants starting at Nieuwkoop - Faber (NF) stage 8, and exposure was terminated at 96 hours. During the exposure and at termination, the number of dead and malformed embryos was counted. The LC50 and EC50 for mortality were determined for each chemical. For TNX, the LC50 was determined to be 57 mg/L, while the EC50 was 53 mg/L. There were no effects of RDX or HMX on survival or development at any of the concentrations tested, up to the limit of water solubility.

9.0 TEST MATERIALS:

Test Chemical name: cyclotetramethylene-tetranitramine (HMX)
CAS number: 2691-41-0
Characterization: Determination of concentration in water samples.
Source: Aldrich Chemical Company

Test Chemical name: cyclotrimethylenetrinitramine (RDX)
CAS number: 121-82-4
Characterization: Determination of concentration in water samples.
Source: Aldrich Chemical Company

Reference Chemical name: FETAX medium was prepared using distilled, carbon filtered water and reagent grade salts (NaCl, 10.7 mM; $NaHCO_3$, 1.14 mM, KCl, 0.4 mM; $CaCl_2$, 0.14 mM; $CaSO_4$, 0.35 mM; $MgSO_4$, 0.62 mM).
CAS Number: Not applicable
Characterization: Determination of pH and conductivity.
Source: City tap water that has been run through reverse osmosis and a de-ionizer to convert it to ultrapure water, with added FETAX salts.

10.0 JUSTIFICATION OF TEST SYSTEM:

Amphibians are currently a major focus of research because of worldwide population declines and occurrence of deformities (Pechmann et al., 1991; Schmidt 1997; Kavlock 1998). Also, recent evidence has suggested that exposure to contaminants increases amphibian's susceptibility to effects of other environmental agents (Burkhart et al., 1999). Thus, the aim of this proposal is to determine the developmental toxicity of RDX and HMX. This study is of vital importance because there is infinitesimal toxicological information on RDX and HMX in general and specifically in amphibians. Large scale manufacture, use, and improper disposal of RDX and HMX are suggested to be a worldwide problem which has led to severe contamination of soil and ground water by this compound and its metabolites. The proposed studies will aid in predicting the impact of RDX and HMX on *Xenopus*. The proposed focus of this study is to expand our knowledge of RDX and HMX contamination using laboratory studies designed to characterize and quantify ecological risks associated with exposure to RDX and HMX in the ecosystem. This research project will address data gaps related to RDX and HMX on amphibian and will provide data for ecological risk assessment as we have successfully accomplished with our perchlorate program.

11.0 TEST ANIMALS:

Species: African clawed frog (*Xenopus laevis*)

Strain: Outbred

Age: Larvae.

Number: Approximately 2400

Source: Bred from captive stocks currently maintained in the laboratory.

12.0 PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:

The test system consisted of laboratory exposures constructed according to the experimental design described below. Glass Petri dishes were labeled with the beaker number, species name, animal use protocol number, project number, test system, and date of hatch. Experimental dishes were labeled with beaker number, project number, date of exposure and date of collection, exposure concentration, and person responsible.

13.0 EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:

The test system consisted of laboratory exposures constructed according to the experimental design described below. Glass Petri dishes were labeled with the beaker number, species name, animal use protocol number, project number, test system, and date of hatch. Experimental dishes were labeled with beaker number, project number, date of exposure and date of collection, concentration, and person responsible.

14.0 METHODS:

14.1 Animal Selection and Receipt

Animals used were selected from in-house breeding colonies. Animals were selected for breeding if they were not previously bred within the last 30 days.

14.2 Assignment of Animals to Study Group and Identification

The adult animals were freeze-branded with liquid nitrogen for identification purposes. Larvae were placed into Petri dishes labeled with test chemical and concentration.

14.3 Animal Husbandry

Water consisted of reverse osmosis water supplemented with 60 mg/L commercially available aquarium sea salts for adults (Goleman et al. 2001). "Water" consisted of a buffer solution specifically formulated for the larvae of this species, according to ASTM (1998). One third of the water was changed at least every other day. Aquaria were covered with plastic mesh to prevent escape. To breed frogs for egg production, adults were injected with 1000 units human chorionic gonadotropin dissolved in sterile water into the dorsal anterior lymphatic sac, and returned to tanks. After injection, one male and one female were placed in a 20 gallon aquarium with a false bottom (plastic-coated hardware cloth) to allow eggs to sink to the bottom without being eaten. Frogs were kept at a water temperature of 23º C (the preferred temperature of this tropical species) at a day/light cycle of 12:12 h light;dark. Water chemistry (pH, dissolved oxygen, ammonia, nitrate, nitrite, temperature) were monitored every other day, using a water quality meter (YSI, Inc.) and a spectrophotometric-based water quality kit (HACH, Inc.).

14.4 Test Material Application

Xenopus larvae were exposed to seven concentrations of RDX or HMX in separate experiments, plus zero control. Eggs (Nieuwkoop -Faber [NF] stages 8-10, Nieuwkoop and Faber 1967) were placed into pre-cleaned Petri dishes and the hatched larvae were allowed to develop for 96 hours while being exposed to toxicants. Dishes were cleaned by washing according to SOP AQ-1-23 "Cleaning Glassware for Use with Xenopus laevis", and all Petri dishes were baked at 250° C for 4 hours before use. For exposures,

dishes were located in a controlled-temperature incubator. The arrangement of the dishes within the incubator were randomized in order to avoid effects due to gradients in light, temperature, volatile chemicals in the laboratory, etc. Arrangement of the dishes within each block was determined by a random number generator. Each block contained at least 1 beaker of each treatment.

The overall experimental design consisted of range finding tests, followed by definitive tests, in which the larvae were exposed to RDX, HMX, and TNX according to the following scheme:

> Range finding tests: 10 larvae/replicate x 1 replicate per treatment x 7 treatments
> Definitive tests: 10 larvae/replicate x 3 replicates per treatment x 7 treatments

Treatments for toxicity tests included control (FETAX medium only) plus 6 concentrations of TNX, and RDX. Only 1 concentration of HMX (water saturation) was used.

14.5 Exposure Verification
At the beginning and end of the exposures, water samples (25 ml) were taken for analysis of chemicals in water. Stock solutions were also analyzed for chemical concentrations in water.

14.6 Food and Water Trace Contamination
Not determined.

14.7 Daily Observations
Each day, all Petri dishes were examined for dead and malformed embryos.

14.8 Euthanasia
At the end of the exposure, all animals were euthanized by immersion in 0.5 g/L MS222

14.9 Sample Collection
Water samples for analysis were collected by siphoning water out of the Petri dishes.

14.10 Sample Storage
Water samples were stored at 4° C until analysis.

14.11 Sample Processing
Water samples were analyzed by HPLC.

14.12 Sample Analysis
TNX 1000 ppm was measured at 952.19 ppm.

RDX at water saturation was measured at 38.7 ppm.

HMX was at water saturation level

14.13 Statistical Analysis

For LC50 and EC50 determinations, the PROC PROBIT procedure of SAS was used to perform profit analysis.

15.0 RESULTS:

For RDX, the concentrations used were 20%, 40%, 50%, 60%, 80%, and 100% of saturated RDX-water. Water saturation was determined to be 38.7 ppm in FETAX. No toxic effects, either mortality or deformities, were noted at the limit of water saturation, so this line of investigation was not continued further.

Saturated HMX-water was used in these experiments. No toxic effects, either mortality or deformities were noted at the limit of water saturation, so this line of investigation was not continued further.

For TNX, the range finding concentrations were 0, 0.001, 0.01, 0.1, 1.0, 10, and 100 PPM (mg/L). For the definitive test, the concentrations used were 0, 40, 45, 50, 55, 60, 65, 70, 75, and 80 mg/L. The LC50 for TNX was 57 mg/L (Fig 1A.). For malformations, the EC50 of TNX was 53 mg/L (Fig. 1B)

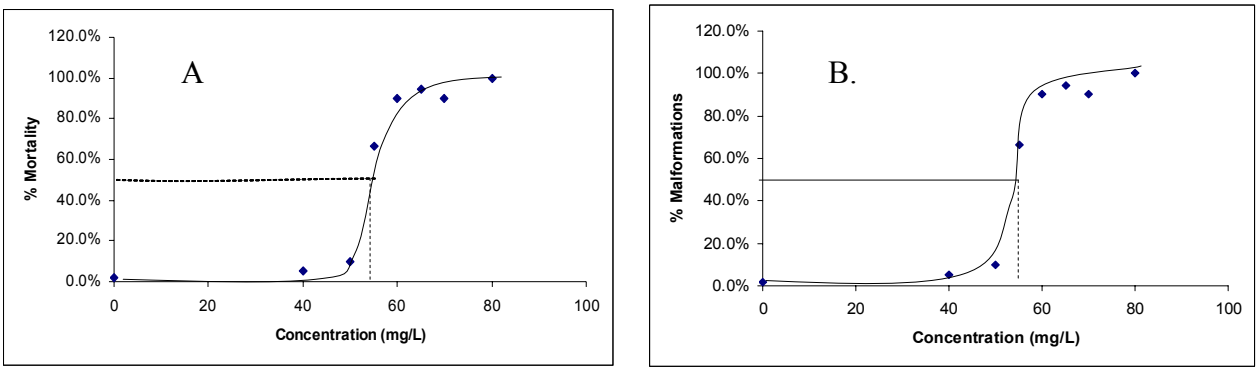

Figure 1. Mortality (A) and % malformations (B) of *Xenopus laevis* exposed to TNX for 96h.

16.0 DISCUSSION:

The results presented above indicate that there is minimal hazard for RDX and HMX at environmentally relevant concentrations, at least for *X. laevis*. TNX did show some acute toxicity, at levels around 55 mg/L. In addition, there were some teratogenic effects close to this concentration as well. Since, all chemicals exhibit teratogenic effects at or near their acute toxicity levels, a "teratogenicity index" has been developed for this assay (TI = LC50/EC50), such that an index greater than 1.5 indicates a teratogenic hazard (ASTM 1998). In this case the teratogenic hazard of TNX is 1.08, indicating no teratogenic hazard for *Xenopus*. In addition, the concentrations of TNX at which effects are seen are

relatively high, and TNX is typically found in the field in anaerobic environments. Thus, it would not be highly likely that amphibians in the field would experience such concentrations. And therefore, the TNX data does not appear to indicate an elevated hazard for *Xenopus*. However, there are several sources of uncertainty for this hazard assessment, and these are as follows: 1) *Acute-chronic extrapolation*: It is currently unknown if there would be greater effects for longer exposures, beginning at the embryonic stage. It is also unknown if chronic exposures would lead to sublethal effects (e.g., effects on immunity, behavior, etc.), that would affect survival in the wild. 2) *Interspecific extrapolation*: The relative sensitivity of *Xenopus* compared to native frog species is currently unknown. 3) *Toxicity of aerobic metabolites* – for reasons that will not be detailed here, the anaerobic degradation product of RDX (TNX) was tested here. However, it is not known if the aerobic degradation product of RDX and HMX are more toxic than the anaerobic degradation products. 4) *Effects of mixtures* – the chemicals used in this study were applied as a single chemical; however, animals in the field are likely to be exposed to mixtures of contaminants. It is currently unknown if there are interactive effects between RDX, HMX, and TNX, or if these chemicals interact with other xenobiotics.

17.0 REFERENCES:

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Newkoop and Faber

TITLE: ENVIRONMENTAL MODELING

STUDY NUMBER: MOD-04-01

SPONSOR: Strategic Environmental and Research Development Program
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CONTRACT ADMINISTRATOR: The Institute of Environmental and Human Health
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RESEARCH INITIATION: October 1, 2002

RESEARCH COMPLETION: August 31, 2005

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GOOD LABORATORIES PRACTICES STATEMENT

This study was conducted in accordance with established Quality Assurance Program guidelines and in the spirit of Good Laboratory Practice Standards whenever possible (40 CFR Part 160, August 17, 1989).

Submitted By:

_____

Kenneth R. Dixon Date

QUALITY ASSURANCE STATEMENT

This study was conducted under The Institute of Environmental and Human Health Quality Assurance Program and whenever possible to meet the spirit of the Good Laboratory Practices as outlined in 40 CFR Part 160, August 17, 1989.

Submitted By:

_____ _____

Quality Assurance Manager Date

1.0 DESCRIPTIVE STUDY TITLE:
ENVIRONMENTAL MODELING

2.0 STUDY NUMBER:
MOD-04-01

3.0 SPONSOR:
Strategic Environmental and Research
Development Program
SERDP Program Office
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4.0 TESTING FACILITY NAME AND ADDRESS:
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5.0 EXPERIMENT START AND TERMINATION DATES:
Start date: October 1, 2002
Termination date: August 31, 2005

6.0 KEY PERSONNEL:
Kenneth R. Dixon, Study Director
Eric P. Albers, Research Assistant
Min Lian, Research Assistant

7.0 STUDY OBJECTIVES/PURPOSE:
Modeling efforts utilized previously developed models to simulate the movement and effects of explosives. A suite of models have been developed to simulate the transport, uptake, and effects of explosives in terrestrial ecosystems (Figure 1). The emphasis in model development to date has been on uptake and distribution of explosives in mammal, bird, reptile, and plant species. Little information has been available on the effects of explosives on reptile species. The lab and field studies in this continuation will provide data that can enhance the effects aspects of the models.

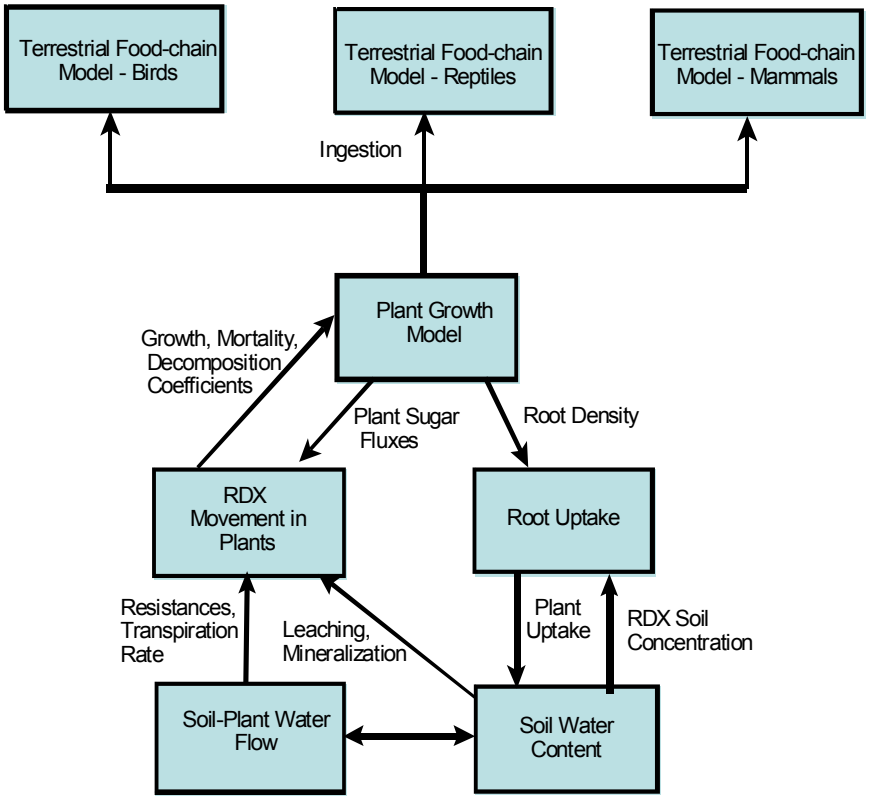

Figure 1. Flow diagram of explosives modeling suite.

We also completed the implementation of the suite of models to provide for large-scale simulations, including estimates of risk for a risk assessment of explosives. This integrated suite of models will be used to assess explosives effects at different contaminated sites.

Small Mammal Model. The small mammal model was adapted from a similar model developed to predict the effects of perchlorate on thyroid activity (Dixon, et al. 2005). Mortality was added as a state variable in the model.

Bird Model. The bird model was adapted from a similar model developed to predict the effects of perchlorate on thyroid activity (Apodaca, et al. 2005). Food items for bird species and the explosives concentration in those items will be added to the model as those data become available from lab and field studies.

Plant Model. Little is known about explosives transport mechanisms in plants. Lab and field studies on explosives exposure in plants will provide data to incorporate more mechanistic transport processes in the plant models. Measured explosives concentrations in different plant

tissues will provide data for model calibration and validation.

8.0 METHODS

Small Mammal Model.

A physiologically based toxicokinetic (PBTK) model was developed to simulate the movement of explosives within small mammals. Contaminant movement was governed by a series of mass-balanced difference equations programmed in Matlab®. Model compartments and blood flow can be seen in Figure 2. Compartments in the small mammal model include blood, heart, brain, fat, kidneys, liver, gut wall, and gut contents (Figure 2). The primary environmental exposure pathway in small mammals is ingestion of explosive-contaminated food and water.

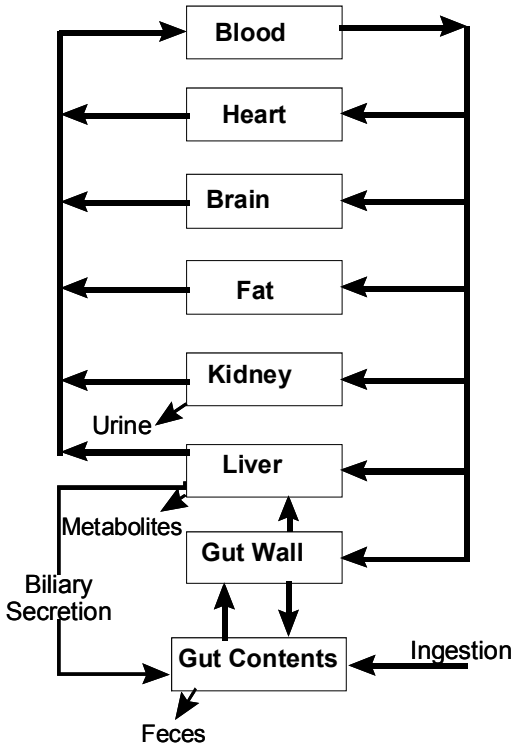

Figure 2. Flow diagram of small mammal PBPK model.

The amount of explosives distribution between venous blood and tissue concentration, $A_{i,t+1}$, is:

$$A_{i,t+1} = A_{i,t} + F_i\left(C_{vb} - \frac{C_i}{P_i}\right)$$

where,
$A_{i,t}$ is the amount of explosives in compartment i at time t, mg,
C_{vb} is the venous blood explosives concentration at time t, mg•L^{-1},
F_i is the blood flow rate into the compartment i, L•h^{-1},
P_i is the explosives partitioning coefficient for compartment i,

Explosives concentration in a compartment is the amount of explosives in the compartment divided by the mass of the compartment:

$$C_i = \frac{A_i}{m_i}$$

where m_i is the mass of compartment, g.

The amount of explosives in the gut, $A_{g,t+1}$, results from the rates of ingestion and elimination of explosives in the interval which can be described by the difference equation:

$$A_{g,t+1} = A_{g,t} + If_t + Iw_t + abs \cdot C_l - aks \cdot C_{gw} - akf \cdot C_g$$

where
C_l = explosives concentration in the liver, µg·g^{-1}
C_{gw} = explosives concentration in the gut wall, µg·g^{-1}
C_g = explosives concentration in the gut, µg·g^{-1}
$If_{i,t}$ = ingestion rate of explosives in food item i at time t, µg·g^{-1} h^{-1}
Iw_t = ingestion rate of explosives in drinking water at time t, µg·g^{-1} h^{-1}
abs = liver absorption rate constant, h^{-1}
aks = gut wall absorption rate constant, h^{-1}
akf = elimination rate constant, h^{-1}

The weight-specific mass ingestion rate of explosives in food, If_t, (µg·h^{-1}) may be written as

$$If_t = \sum_{i=1}^{m} p_i \times Cf_i \times \upsilon_i$$

where
p_i = proportion of total diet contributed by item i at time t
Cf_i = consumption rate of food item i , g·h^{-1}

v_i = explosives concentration in food item i , $\mu g \cdot g^{-1}$

Consumption rate is a function of body weight (USEPA 1993):

$$Cf = 0.398 \cdot W^{0.850}$$

where W = consumer body weight, g

Similarly, for ingestion of explosives in water, Iw_t ($\mu g \cdot h^{-1}$) is:

$$Iw_t = Cw \cdot \upsilon$$

where
Cw = consumption rate of water, $L \cdot h^{-1}$
v_i = explosives concentration in water, $\mu g \cdot L^{-1}$
W = consumer body weight, kg

Parameter estimation

RDX LD50 values, obtained from data reported for single gavage doses, ranged from 71 to 118 mg/kg in rats and from 86 to 97 mg/kg in mice (Army 1978, 1980). Flow rates and tissue volumes were obtained from Brown, et al. (1997). Partitioning coefficients were calculated from data in Schneider, et al. (1978).

Bird Model. PBTK Model Description. A physiologically based toxicokinetic (PBTK) model was developed to simulate the movement of explosives within birds. Contaminant movement was governed by a series of mass-balanced difference equations programmed in Matlab®, similar to those in the mammal model. Model compartments and blood flow can be seen in Figure 3. Distribution was assumed to be flow-limited, i.e. chemical equilibrium existed between the tissues and blood leaving the compartment. An ingestion term was included to allow for the incorporation of multiple food sources of varying levels of toxicity.

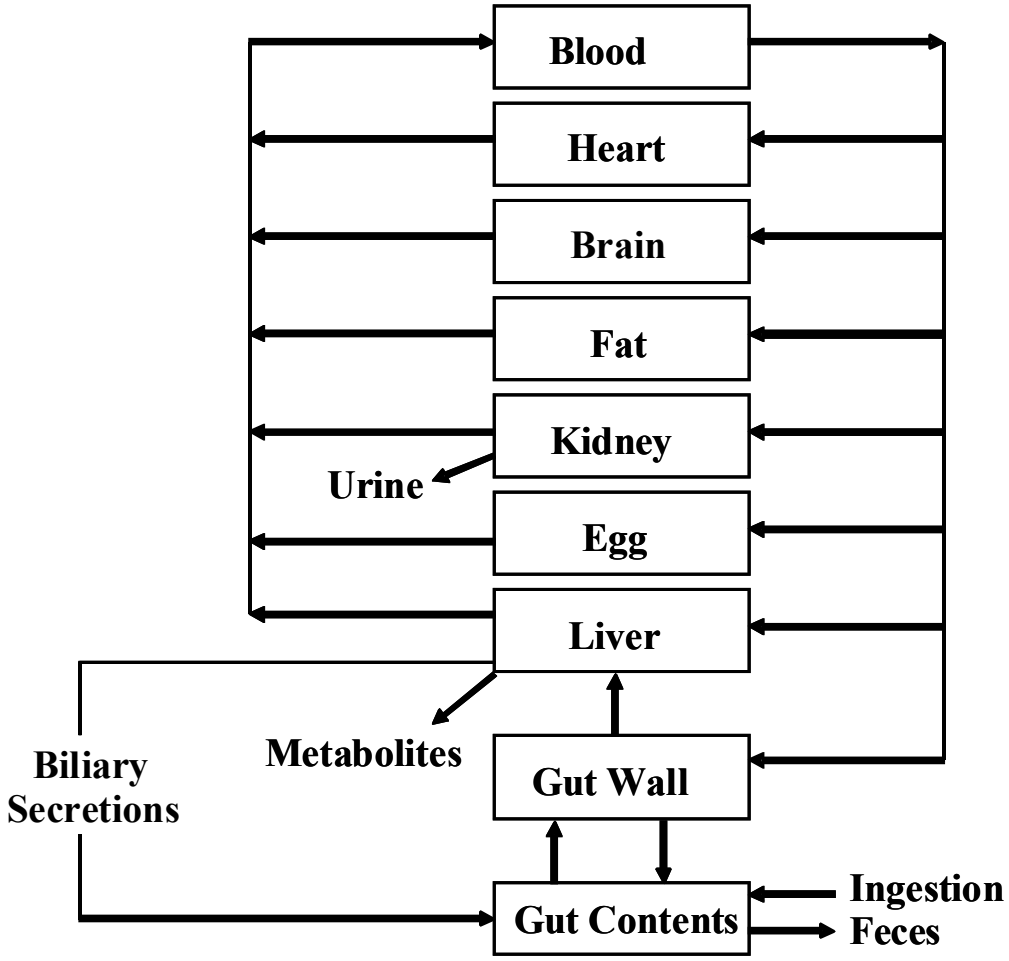

Figure 3. Flow Diagram of the PBTK model for explosives uptake in birds.

Plant Model. The model simulates explosives uptake in terrestrial macrophytes and was programmed in Matlab using difference equations. To simulate and predict the uptake and transport of explosives in various terrestrial and aquatic plants, we developed new uptake and distribution components that are specific to explosives and modified the CERES model (Dixon, et al. 1978) by incorporating these new components (Figure 4). Additionally, an internal hydrological component was added to simulate environmental soil and water conditions.

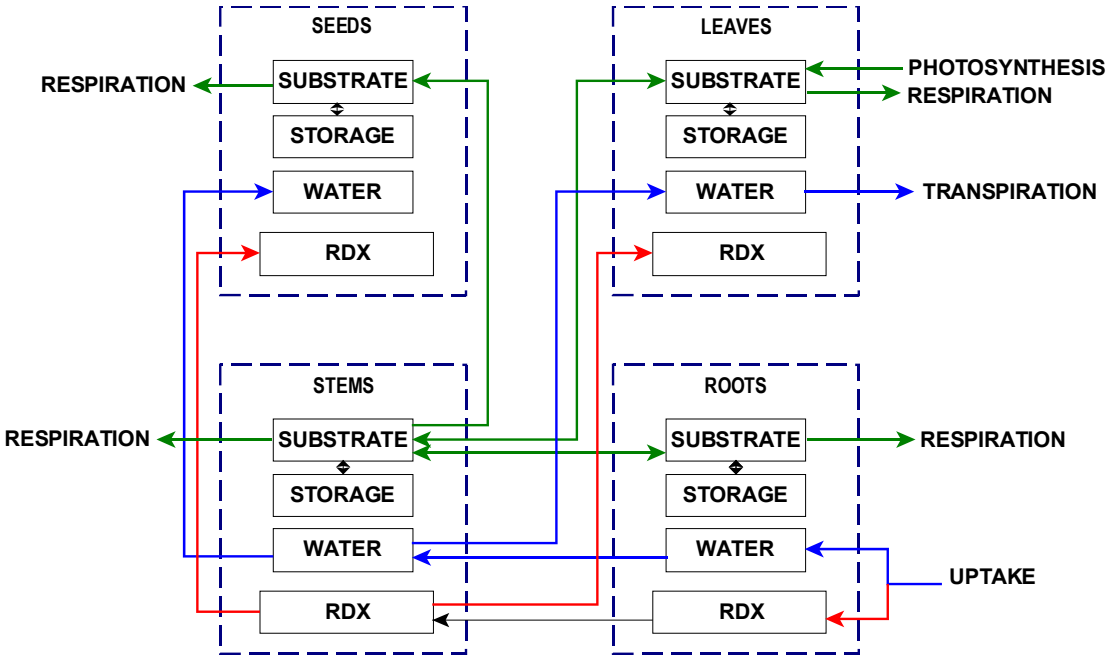

Figure 4. Flow diagram of plant uptake model.

For the macrophyte model plant growth governing equations, we used those described by Dixon, et al. (1978).

The plant's water uptake is the product of the plant's ability to take up water, its leaf area, and the mass of water available to the plant in its growing soil horizon:

$$U_t^i = f \cdot L_t^{'} \cdot SM_A$$

where,

U_t^i = incremental water uptake at time t
f = water flow constant (h^{-1})
L'_t = Leaf Area Index
SM_a = Mass of water in Soil Horizon A ($g \cdot m^{-2}$ land area/hour)

Distribution of water and explosives between compartments is defined by the difference in water and explosives between compartments:

$$F_{ab} = \begin{cases} (S_a - S_b)/r_{ab} & t_1 < t \le t_4 \\ 0 & otherwise \end{cases}$$

where,
F_{ab} = flux from compartment a to compartment b ($g \cdot m^{-2}$ land area/hour)
S = amount of water in a given compartment ($g \cdot m^{-2}$ land area)
r_{ab} = water flux constant
t_1 = starting day of the growing season
t_4 = ending day of the growing season

The amount of explosives in individual compartments is defined by:

$$M_a = F_{ab} \cdot C_{\mathrm{RDX}}$$

where

M_a = mass of explosives in compartment a at time t ($\mu g \cdot m^{-2}$ land area)
F_{ab} = flux of water between the two involved compartments
C_{RDX} = RDX concentration in the incoming water

The ratio of the amount of explosives in the compartment to the biomass (wet weight) of the compartment determines the explosives concentration:

$$Q_{a,t} = \frac{M_{a,t}}{B_{a,t} + W_{a,t}}$$

where,
$Q_{a,t}$ = concentration of explosives in compartment *a* at time *t* ($\mu g \bullet g^{-1}$)
$M_{a,t}$ = amount of explosives in compartment *a* at time *t* ($\mu g \bullet m^{-2}$)
$B_{a,t}$ = biomass in compartment *a* at time t ($g \bullet m^2$)
$W_{a,t}$ = mass of water in compartment a at time *t* ($g \bullet m^2$)

Plant biomass is calculated by summing the soluble and insoluble photosynthate fractions (Dixon, et al. 1978):

$$B_a = S_a + ST_a$$

where,
$B_{a,t}$ = biomass of compartment *a* at time *t* ($g \bullet m^2$)
$S_{a,t}$ = sugar substrate in compartment *a* at time *t* ($g \bullet m^2$)
$ST_{a,t}$ = plant storage tissue in compartment *a* at time *t* ($g \bullet m^2$)

Model Assumptions:

- transport between leaves and stems occurs from the time of bud formation to the time of abscission.
- transport between stems and fruits occurs from the time of net photosynthesis to the time of abscission.
- transport between the stems and roots is assumed to occur throughout the year.

9.0 RESULTS

Small Mammal Model.

The small mammal model was calibrated using data on the RDX distribution in different organs in the rat (Schneider, et al. 1978). The simulated results were compared with the observed values from experiments with chronic ingestion of RDX saturated drinking water at 50-70 µg/ml *ad libitum* for 90 days (Figure 5). A second experiment with a chronic oral administration of RDX was used to test the model's mortality function. We used data on rat dams that were fed 20 mg/kg/day of RDX during gestation that had mortality rates of 30% (Army 1980, 1986). These results are shown in Figure 6.

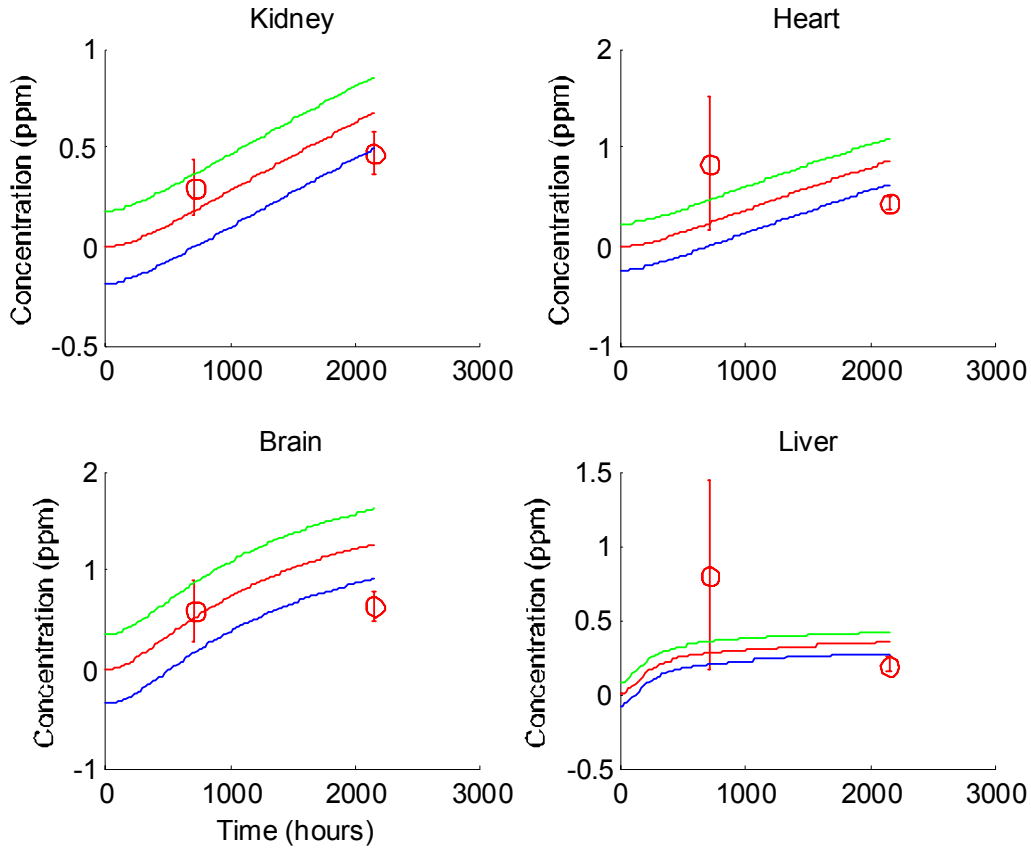

Figure 5. Observed and predicted RDX concentrations in kidney, heart, brain and liver compartments. Red lines are means. Green and blue lines are upper and lower 95% confidence limits respectively. Red circles with error bars are observed means ±S.E. (data from Schneider, et al. 1978).

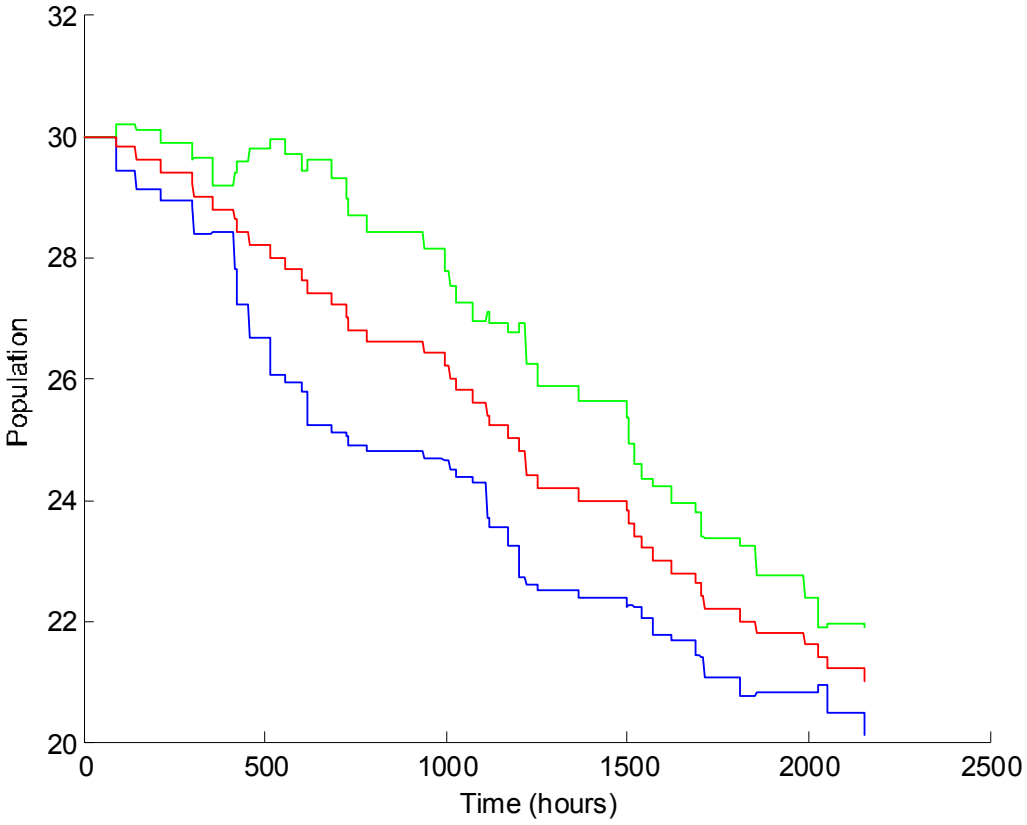

Figure 6. Predicted population size following daily gavage of 20mg/kg of RDX in rats. Red line is the mean. Green and blue lines are upper and lower 95% confidence limits respectively. Mortality data are from Schneider, et al. (1978).

Schneider, et al. (1978) reported mortality in eight of thirty rats between days 42 and 77 in the 90 day chronic oral administration experiment. Our model predicted similar mortality but continuously throughout the simulation.

Bird Model.
The model was calibrated using data from Gogal, Jr., et al. (2003). The model will be calibrated further using data collected in the laboratory.

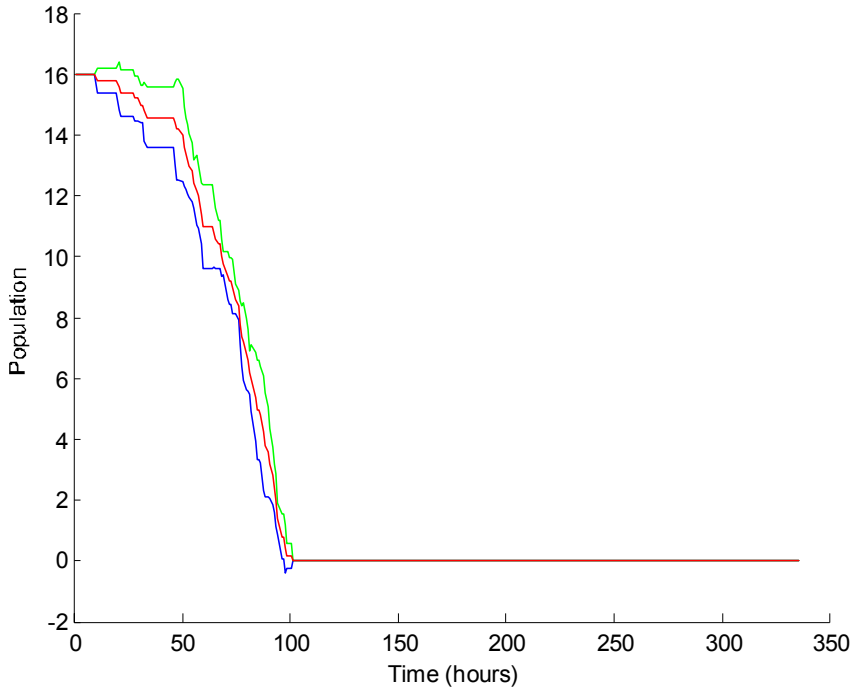

Figure 7. Simulated lab bobwhite population of 16 birds following an acute lethal dose of 945 mg RDX/kg. Mortality data are from Gogal, Jr., et al. (2003). Red line is the mean. Green and blue lines are upper and lower 95% confidence limits respectively.

In this 14-day lab experiment, all birds died within 72 hours at doses greater than 630 mg/kg. The simulation showed similar results with a dose of 945 mg/kg causing 100 percent mortality by 100 hours. In a second 14-day lab experiment, quail were exposed to feeding mixtures ranging from 0 to 420 mg/kg. No birds died over the course of the feeding trial and no birds died in the simulation. Quail lost weight and decreased food consumption with increasing RDX exposure. At the 280 mg/kg exposure level, the average weight loss was approximately 25g. In the simulation at 280 mg/kg, we assumed a similar weight loss at a constant rate over the 14 days of exposure starting at day 7 (hour 168) (Figure 8). Food consumption followed a similar pattern because food consumption is a function of body weight (page 8) (Figure 9).

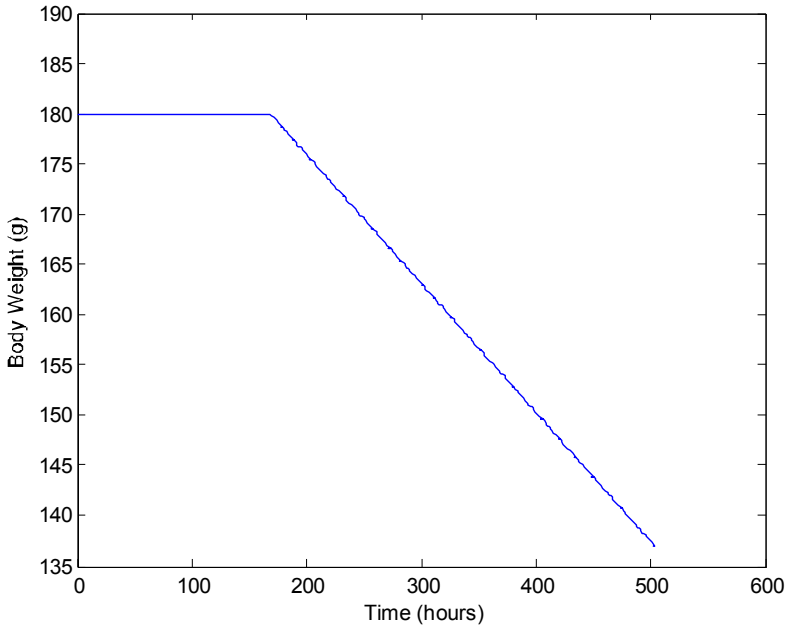

Figure 8. Predicted body weight (g) in a 180g bobwhite with RDX concentration of 50.4 g/kg in food starting at day 7 (hour 168).

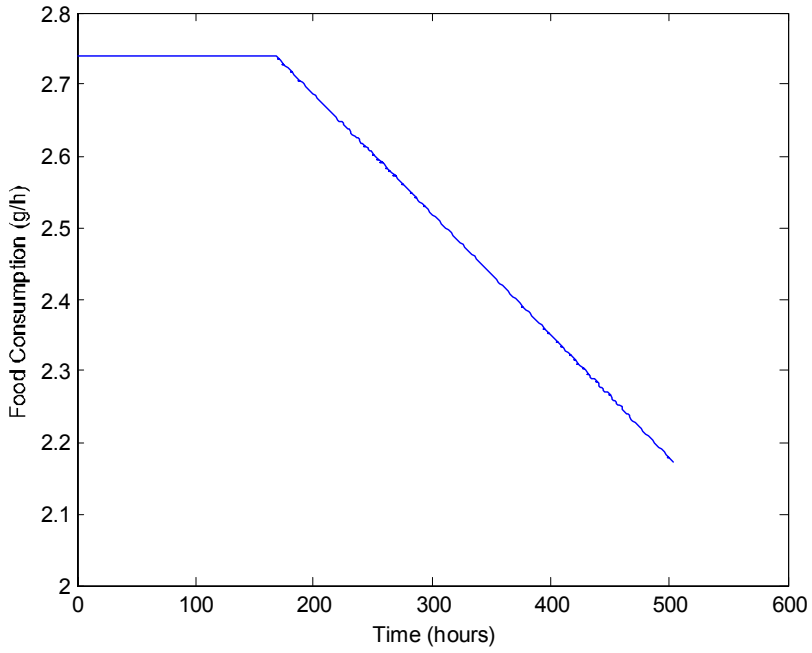

Figure 9. Predicted food consumption (g/h) in a 180g bobwhite with RDX concentration of 50.4 g/kg in food starting at day 7 (hour 168).

Plant Model.

There are few data available on the partitioning of explosives in plant parts. We used data from a study of RDX in plants (Price et al. 2002). Measured leaf and fruit biomass were 723±34.0 and 22.6±8.70 respectively (Figure 10), using a soil concentration of 7.68±0.18 mg/kg from the same study. Measured RDX concentrations in tomato fruits were approximately 10 mg/kg (Figure 12). The predicted explosives concentration was adjusted to fit the observed data by reducing uptake from the soil water and reducing the flow into leaves and fruits.

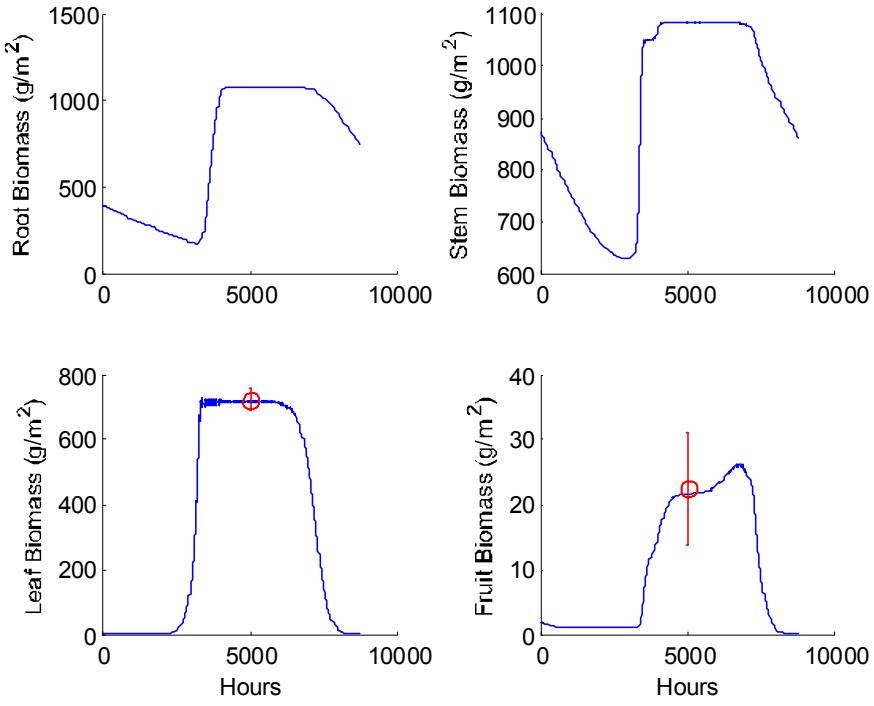

Figure 10. Predicted biomass in tomato plant parts (in blue). Observed biomass shown as mean ± standard error (in red).

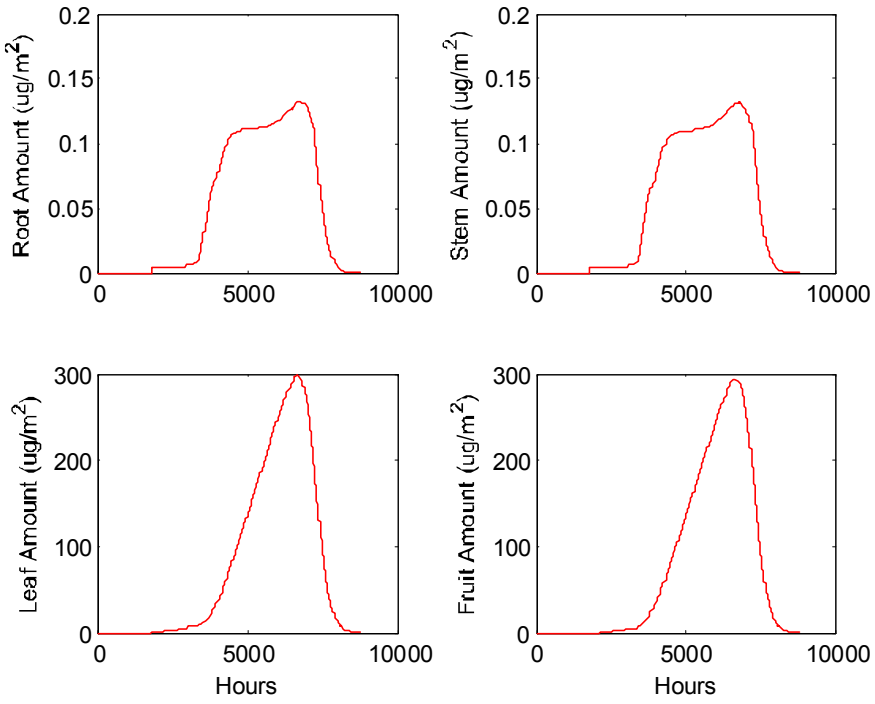

Figure 11. Predicted RDX mass in tomato plant parts.

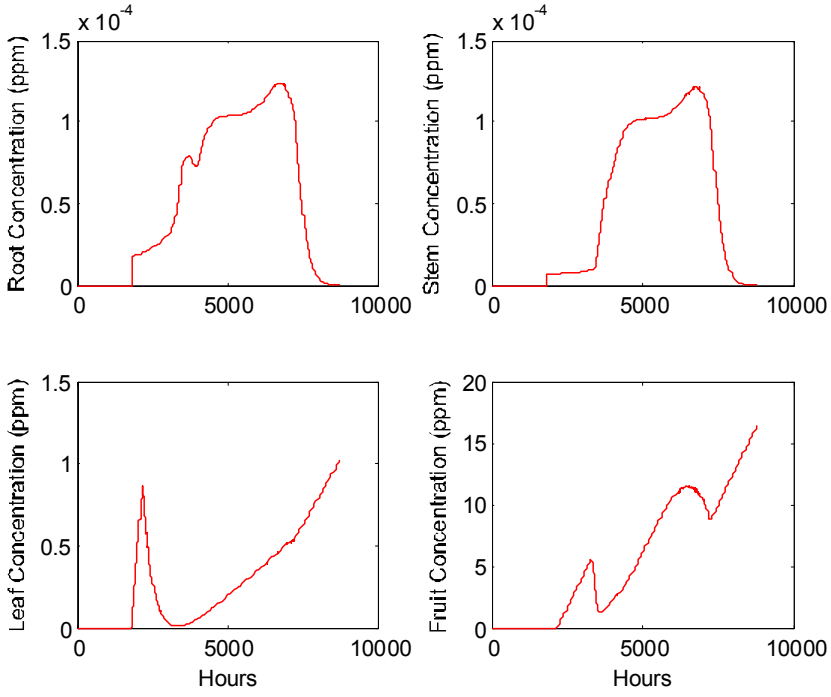

Figure 12. Predicted RDX concentration in tomato plant parts.

10.0 DISCUSSION

Small Mammal Model.
In the 90 day chronic oral administration experiment (Schneider, et al. 1978), the experimental rats became lethargic, lost weight, developed a rough hair coat, and exhibited a blood-tinged exudate around the external nares immediately prior to death. This sub-acute morbidity should be added to the model to account for the delayed mortality.

Bird Model.
Explosives levels in food items were based on limited data. Because of a lack of lab and field data for calibration and verification we are unable to test these terms at this time. Appropriate data on ingestion rates, feeding preference, and food item contamination are needed. Estimates of LD50 and LD10 also are needed to parameterize the dose-response function in the model.

Plant Model.
The vascular plant model was developed under the assumption that water is the driving force behind the uptake and distribution of explosives in plants. Because the model predicts tissue concentrations that are in line with laboratory values, it is reasonable to assume that water movement in plants is an important driving force in the uptake and distribution of explosives.

The model also indicates that explosives are capable of bioaccumulation in the leaves and fruits of exposed plants. If this result is true, there is significant potential for trophic transfer of explosives if wildlife and humans consume exposed plants. Although parameter estimates were based on calibration with lab data, direct parameter estimation may improve the accuracy of the model predictions.

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A FINAL REPORT

HMX Toxicity in Birds and Reptiles

STUDY NUMBER: HMX-04-01

SPONSOR: Strategic Environmental and Research Development Program
SERDP Program Office
901 North Stuart Street, Suite 303
Arlington, VA 22203

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RESEARCH INITIATION: July 2003

RESEARCH COMPLETION: June 2005

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GOOD LABORATORIES PRACTICES STATEMENT

This study was conducted in accordance with established Quality Assurance Program guidelines and in the spirit of the Good Laboratory Practice Standards whenever possible (40 CFR Part 160, August 17, 1989).

Submitted By:

_____ _____

Philip N. Smith Date

QUALITY ASSURANCE STATEMENT

This study was conducted under The Institute of Environmental and Human Health Quality Assurance Program and whenever possible to meet the spirit of the Good Laboratory Practices as outlined in 40 CFR Part 160, August 17, 1989.

Submitted By:

_____ _____

Quality Assurance Manager Date

2.0 **DESCRIPTIVE STUDY TITLE**: HMX Toxicity in Birds and Reptiles

2.0 **STUDY NUMBER**: HMX-04-01

3.0 **SPONSOR:**
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4.0 **TESTING FACILITY**: The Institute of Environmental and Human Health
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TEST SITE: The Institute of Environmental and Human Health
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5.0 **PROPOSED EXPERIMENTAL START & TERMINATION DATES**:
Start Date: July 2003
Termination Date: June 2005

14.0 **KEY PERSONNEL**:
Dr. Philip N. Smith, Co-Project Manager
Dr. Scott T. McMurry, Co-Project Manager
Dr. Tina Brunjes. Post Doctoral Researcher
Mr. Scott Severt, Research Technician
Ms. Lisa Perlmutter, research technician
Mr. Ryan M. Bounds, Quality Assurance Manager
Dr. Ronald J. Kendall, Testing Facility Management

15.0 STUDY OBJECTIVES / PURPOSE:

- To expand the database on HMX to include exposure and toxicity data on birds and reptiles. We have found no data on birds or reptiles with the exception of a recent study by the Army on the approximate acute toxicity of HMX in quail (*Colinus virginianus*),

which focused on lethality following oral dosing with HMX (Gogal et al., 2001; Army, 2001).

- To assess HMX acute toxicity in a new avian species, the red-winged blackbird (*Agelaius phoeniceus*), and assess HMX transfer into eggs and subsequent effects on hatching, and post-hatching growth, development, and survival in quail. Our proposed work on quail differs considerably from that conducted previously for HMX. Our design addressed questions of transfer to eggs and subsequent effects on offspring.

- To assess acute toxicity of HMX and subsequent effects on reproduction in a reptile model, the green anole. Data is lacking on HMX toxicity in reptiles and has been identified as a data gap for this compound (Army, 2001). Baseline studies on these taxon would increase the breadth of our knowledge on the toxicity of HMX among vertebrates, and provide useful information for site managers charged with assessing and managing risk of HMX to resident wildlife at HMX-contaminated sites.

16.0 **TEST MATERIALS**:

Test Chemical name: octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX)
CAS number: 2691-41-0
Characterization: explosive
Source: Accurate Energetics

17.0 JUSTIFICATION OF TEST SYSTEM:

Numerous military training exercises require detonation of live or training munitions which can release residual chemicals into the environment. Energetic compounds such as octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) are commonly found in soils and other environmental matrices at military training installations (Talmage et al., 1999). Heretofore, the ecotoxicological issues involving HMX contamination on military training installations have not been well defined or studied (see USACHPPM 2001). In this sub-project, our goal was to fill ecotoxicological data gaps identified by the U.S. Army Center for Health Promotion and Preventive Medicine (USACHPPM) for HMX specifically related to birds and reptiles.

To our knowledge, there is no information on the toxicity of HMX to wildlife species in the peer-reviewed literature. Published studies have described the effects of HMX in laboratory mammalian models, including rats, mice, and rabbits. Results of these studies indicate variable responses in lethal and sublethal effects. HMX is toxic to both mice and rats in an apparent sex-and species dependant manner. Death was noted in rats during at 14 day study at 9,000 ppm in males and 1,000 ppm in females (Army 1985a). Mice were reversed in the sensitivity of males and females, and responded at lower doses. Death was observed in male mice at 300 ppm and females at 800 ppm (Army 1985b). Other toxic responses in rats and mice were noted in longer studies, including reduced weight gain and food consumption, hematological alterations, liver and kidney pathology (Army 1985c and d). These observations varied among doses and rodent species, but overall, HMX is typically considered of low toxicity in mammals.

Results of HMX studies in rodents also showed the rapid nature of elimination of the compound. HMX in rodent plasma after 13 weeks of exposure via food was negligible and did not change with dose levels (Army 1985e). Single dose studies with ^{14}C-HMX demonstrate this nature (Army 1986). Specifically, 85% of a single dose of HMX at 500 mg/kg in rats was eliminated in feces in 4 days (70% in mice). Similarly, 61% of HMX administered IV to rats was eliminated in urine in 4 days. HMX was rapidly metabolized to very polar metablolites and appears poor at accumulating in tissue after oral dosing. Concentrations of HMX were highest in liver, kidney, and brain.

Given that the current data on the toxicity of HMX in rodents can be used as a reasonable predictor of exposure and toxicity in wild mammals, this study was conducted to expand the database on HMX to include exposure and toxicity data on birds and reptiles. Quail are routinely used in toxicological evaluations as a recognized avian model. Blackbirds can also be cultured in laboratory settings and have proven to be a sensitive avian model in a variety of toxicity studies. Lizards have recently been proposed as a viable reptile model for toxicity studies given their wide distribution, ability to be cultured in the laboratory, and sensitivity to endocrine disruption (Talent et al. 2002). This study will provide needed information for developing screening benchmark levels for these animal species, and determine if these and related species are at risk to toxicity from HMX at DoD training bases. In addition, results from this study will provide the foundation needed to determine if additional research is warranted, and if so, provide the guidelines for designing additional studies.

18.0 **TEST ANIMALS**:

Species: red-winged blackbird (*Agelaius phoeniceus*), quail (*Colinus virginianus*), green anoles (*Anolis carolinensis*)

Strain: quail (laboratory); green anoles and red-winged blackbirds (free-living)

Age: quail adults and hatchlings; red-winged blackbirds; green anoles (hatching year or after hatching year)

Number: approximately: quail (100); red-winged blackbirds (20); anoles (80)

Source: Quail (commercial breeder); blackbirds (captured from Lubbock, TX); anoles (commercial vendor)

19.0 **PROCEDURE FOR IDENTIFYING THE TEST SYSTEM**:

All animals were placed in standard cages and each cage was labeled with a note card containing the appropriate identification information for the animal. Birds and anoles were placed into individual labeled cages containing the appropriate identification information for the animal on the front of the cage. Collected samples were placed in individually labeled bags/containers and stored appropriately according to TIEHH SOP

IN-3-02.

20.0 EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:

For the LD_{50} portion of the study we used The Organization for Economic Cooperation and Development (OECD) Guideline for Testing of Chemical #425 for Acute Oral Toxicity – Up-and-Down Method. This method has been developed specifically with the intent of producing statistically valid estimates of acute lethality with the absolute minimum number of animal subjects.

For the reproductive portions of the avian and reptilian studies, multiple treatment groups were established at increasing dosages of the test chemical (HMX). In all studies, a control group was included (no HMX exposure) to evaluate experimental treatment effects. All animals within each experiment were cared for similarly to insure no bias. For example, treatment groups were placed randomly on racks so that no one treatment group was sequestered from others. However, extensive effort was extended to insure that no animals were exposed to any treatment matrix not intended for their particular treatment group. All sample collection, processing, and data collection was done in random order with proper measures taken to eliminate cross-contamination of samples. Additional approaches for bias control are included in the methods section below.

21.0 METHODS:

LD_{50} of red-winged blackbirds and quail (2004)

Blackbirds were captured with mist nets from local wild populations and held in individual cages for a 7 to 14 day acclimation period prior to experimental manipulation. Quail were purchased from a commercial vendor and housed according to standard procedures in indoor facilities (to facilitate reproductive studies). Quail were also acclimated for 7 to 14 days prior to any experimental manipulation. All birds were provided with water and commercial avian chow *ad libitum*. All birds were weighed and provided with unique identification leg bands upon acquisition. Any birds that appeared sick (lethargic, losing weight, not eating) during the acclimation period were excluded from the experimental trials. LD_{50}s were then determined using the up-and-down method described in brief below. All birds were dosed with HMX via oral gavage with polyethylene glycol (PEG) as a carrier. Control birds were gavaged with carrier only.

LD_{50} of green anoles (2004)

Initially, it was important to note the acute effects of HMX on the test species. The oral LD_{50} up-and-down method was used to estimate acute toxicity of HMX (OECD 2001). Twelve adult anoles (6 male and 6 female) were gavaged with 2,000 mg/kg of an HMX/polyethylene glycol (PEG) solution and observed for toxicosis for 14 days (2-15 August 2004). An additional female anole was gavaged with vehicle only and served as a control for the experiment. Only one of the HMX-dosed lizards died (male), likely due to factors unrelated to HMX. Given these results, the oral LD_{50} for the green anole was estimated to be >2,000 mg HMX/kg body weight.

General Procedures

Administration of doses

HMX was administered in a single dose by gavage using a stomach tube or a suitable intubation cannula. In the unusual circumstance that a single dose is not possible, the dose may be given in smaller fractions over a period not exceeding 24 hours.

Animals were fasted prior to dosing. Following a period of fasting, the animals were weighed and the test substance administered. The fasted body weight of each animal was determined and the dose calculated according to the body weight.

Limit test at 2000 mg/kg

One animal was dosed at 2000 mg/kg. If the animal died the main would have been done to determine the LD50. When the animal survived, four additional animals were dosed sequentially so that a total of five animals were tested. If three animals died, the limit test was terminated and the main test performed. The LD50 was determined to be greater than 2000 mg/kg if three or more animals survived.

Animals were observed individually at least once during the first 30 minutes after dosing, periodically during the first 24 hours (with special attention given during the first 4 hours), and daily thereafter, for a total of 14 days.

HMX transfer to quail eggs (2004)

Eleven quail were randomly assigned to one of four groups: 0, 25, 125, and 250 mg/kg. HMX was dissolved in acetone and was incorporated into quail food at the concentrations listed above. Food was provided to all treatment groups *ad libidum*. Eggs were collected daily and frozen until residue analysis.

HMX effects on quail survival and development (2005)

Thirty breeding pairs of quail were purchased from an independent commercial breeder (Stephenson Game Farm, Riverside, TX, USA). Each pair was housed in a 25.4 cm x 60.9 cm cage in a 30-section, galvanized-steel battery breeding pen (Georgia Quail Farm, Savannah, GA, USA) throughout the study, including an 8-week acclimation period. Birds were given distilled/deionized water and feed (Bluebonnet Feeds, Ardmore, OK, USA) *ad libitum* throughout the acclimation period and during dosing. Throughout the study, temperature was maintained at 23.0 ± 2.0ºC, relative humidity was maintained at 50.0 ± 5.0%, and the light cycle was 16:18h light:dark. Fresh feed was provided every third day, at which time litter pans were cleaned, and the quantity of waste feed measured.

Dosing began once all pairs began laying eggs. At the onset of dosing, each bird was weighed and banded, and birds were weighed once weekly for the duration of dosing. Twenty-eight pairs of quail were equally distributed among four HMX treatments (a zero, low, medium, and high dose) determined from LD_{50} results. The low dose concentration was 12 mg/kg, the medium dose concentration was 53 mg/kg, and the high dose concentration was 109 mg/kg. An additional pair was randomly selected and placed in

the control group. Adult quail were dosed with HMX via food. Feed was treated using HMX-acetone, which was sprayed on the feed as it was agitated in a mechanical feed mixer. Birds in the control group were given feed treated with a similar quantity of acetone only. Agitation was continued for 10 minutes after the application to facilitate evaporation of the acetone. We prepared feed in 4.5 kg batches, which were tested after 24 hours to insure correct dose, and stored in plastic tubs in a freezer at 5.0 ºC.

Eggs were collected daily from all females, marked with the date and pen number in pencil, weighed, and immediately placed in a Profi-I forced-air incubator (Lyon Electric Company, Inc., Chula Vista, CA, USA). The temperature in the incubator was maintained at 38.0 ± 2.0ºC and the relative humidity was 55.0 ± 5.0%. Eggs were candled after 10 days to determine fertility and viability. After 20 days, eggs were transferred to a Profi-IH forced air hatcher (Lyon Electric Company, Inc., Chula Vista, CA, USA). Eggs which failed to hatch after 4 days in the hatcher were candled to determine during which week of incubation death occurred.

HMX deposition into reptile eggs and reproductive toxicity (2004)

Thirty-four adult female anoles were randomly divided into three dose groups based on the estimated LD_{50} value: control (0 HMX), low (25% of LD_{50}, or 500 mg/kg HMX), and high (50% of LD_{50}, or 1,000 mg/kg HMX). Anoles were dosed with HMX via live crickets. The delivery method used consisted of injecting the appropriate amount of HMX/PEG solution into a cricket, which was immediately offered to the anole. This procedure was conducted 3 times per week for 8 weeks, beginning 24 August 2004 and ending 16 October 2004. Doses were adjusted every two weeks based on changes in body weight of individuals. Each anole was given three opportunities to eat a dosed cricket on any given dose day. If the anole voraciously attacked and ate the dosed cricket, or refused to eat the dosed cricket completely after three attempts, another undosed, vitamin-fortified cricket was offered to the anole to supplement the diet. Otherwise, individuals were allowed to eat 4 crickets per week (3 dosed, 1 not dosed). Typically, a maximum of 4 crickets were consumed per week by any individual. Twenty eight eggs were collected from the three dose groups. Of these, 14 were frozen for residue analysis, with the remaining 14 incubated until hatching.

15.0 RESULTS

LD_{50} of red-winged blackbirds and quail (2004)

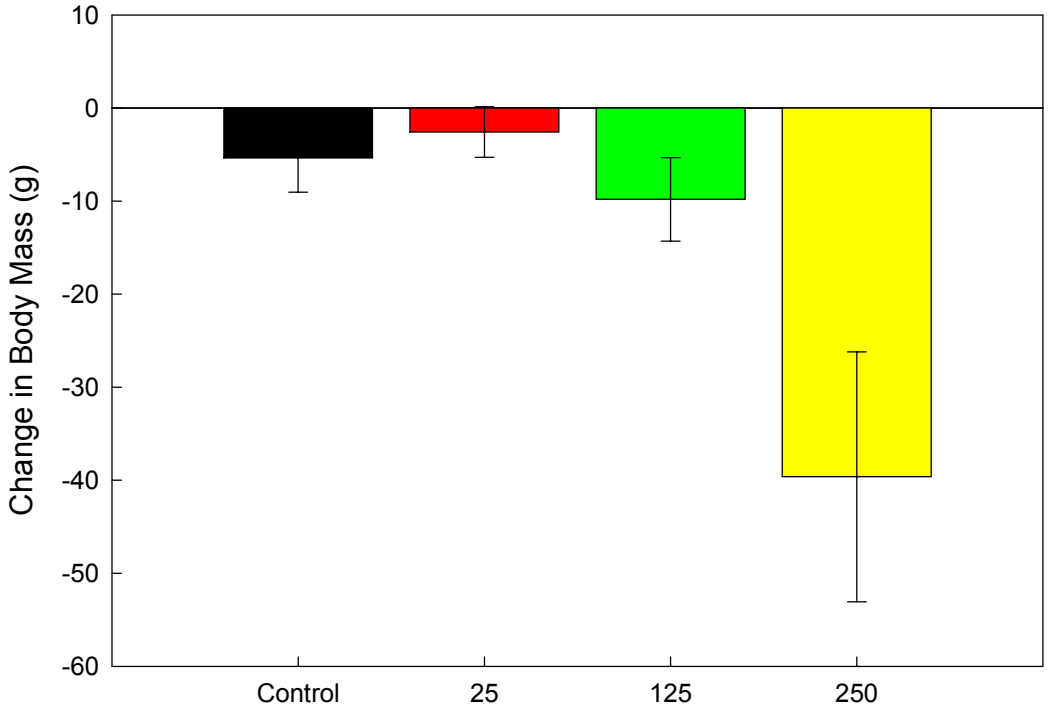
Northern Bobwhite Quail were purchased from T.A.L.A. Kennels & Gamebird Hunting Preserve in Lockney, TX. Upon receipt, the animals were housed in the animal care facility located on the Texas Tech University campus. LD50 tests were conducted in mid August 2004. The late start was due to difficulties in obtaining the test material (HMX). A limit Test was conducted as the initial step in determination of acute lethality in this species following OECD guidelines. Five quail and red-winged blackbirds were dosed at 2,000 mg/kg via gavage and observations recorded. All birds were alive and active following dosing, and all survived to the termination of the test. The LD50 was thus determined to be > 2,000 mg/kg for red-winged blackbirds and bobwhite quail.

LD_{50} of green anoles (2004)

Twelve adult anoles (6 male and 6 female) were gavaged with 2,000 mg/kg of an HMX/polyethylene glycol (PEG) solution and observed for toxicosis for 14 days (2-15 August 2004). An additional female anole was gavaged with vehicle only and served as a control for the experiment. Only one of the HMX-dosed lizards died (male), likely due to factors unrelated to HMX. Given these results, the oral LD_{50} for the green anole was estimated to be >2,000 mg HMX/kg body weight.

HMX transfer to quail eggs (2004)

The egg transfer portion of the study began in late September 2004. Dose-dependent reductions in body mass were observed among quail dosed with HMX in food (Fig. 1). These findings are similar to those of Johnson et al. (2005) who observed severe food avoidance in bobwhites given food containing HMX.



minal concentrations of 0 (control), 25, 125, and 250 mg/kg.

In addition to weight loss, we observed reductions in egg laying related to HMX exposure. Quail in the high and medium dose groups (250 and 125 mg/kg, respectively) laid fewer eggs than quail in the low (25 mg/kg) and control treatment groups (Fig. 2). Although this appeared to be a treatment-related effect, it was likely due to reductions in food intake rather than a toxic mechanism. Egg production is energetically costly; therefore quail consuming less food and losing weight would not be expected to maintain egg production.

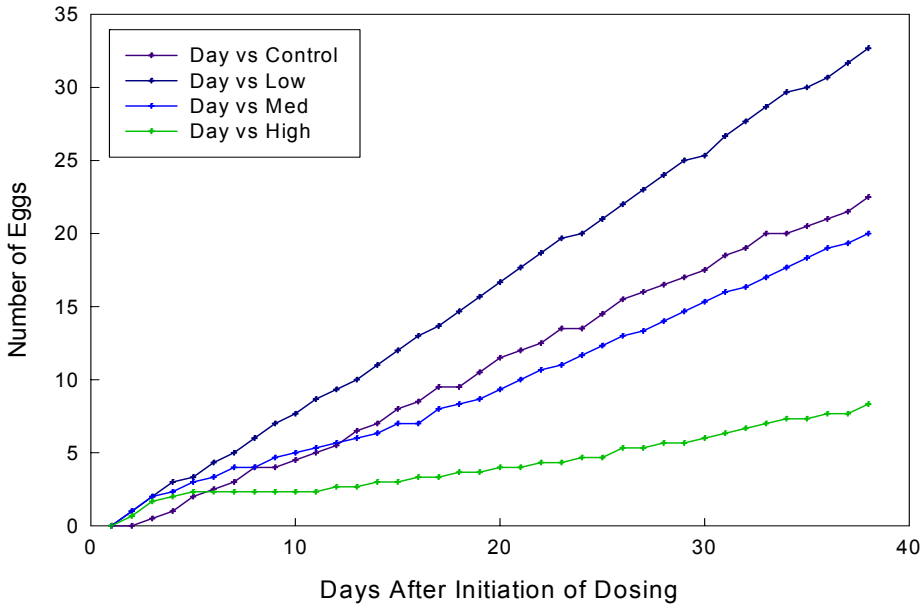


Figure 2. Cumulative egg laying distributions (normalized for number of birds in treatment) for bobwhite quail dosed with HMX in food at nominal concentrations of 0 (control), 25 (low), 125 (med), and 250 mg/kg (high).

HMX was found eggs from all quail in the deposition study including controls. The occurrence of HMX in control eggs was likely due to analytical error. Few control eggs contained HMX, but overall means indicate that HMX was present in at least one control egg from each bird in the control group. However, the mean concentrations in these eggs were orders of magnitude lower than those found in the HMX-treatment group eggs. HMX was apparently readily deposited from female quail into eggs (Table 1).

Table 1. Mean (and standard error; SE) number of eggs (n) containing HMX and mean concentrations of HMX in eggs from adults dosed via food at nominal concentrations of 0 (control), 25 (low), 125 (med), and 250 mg/kg (high).

Quail ID	Mean HMX (ng/g)	SE	*n*
control 1	125.55	39.70	9
control 2	118.37	23.67	25
low 1	1730.53	339.38	26
low 2	1246.42	249.28	25
low 3	1463.33	311.98	22
med 1	7167.01	1433.40	25
med 2	5384.01	1623.34	11
med 3	6657.05	1664.26	16

high 1	3949.06	2279.99	3
high 2	14350.37	3587.59	16
high 3	19846.11	9923.06	4

Concentrations of HMX in eggs among treatment groups were quite variable. Variability was likely due to fluctuations in timing of exposure (food consumption) related to oogenesis. Mean concentrations of HMX in each treatment group were dose-dependent, with the 250 mg/kg group eggs containing the highest concentrations of HMX (Fig. 3). Of particular interest was the rate at which HMX was apparently depurated into eggs. HMX was detected in eggs very quickly (within days) following initiation of dosing.

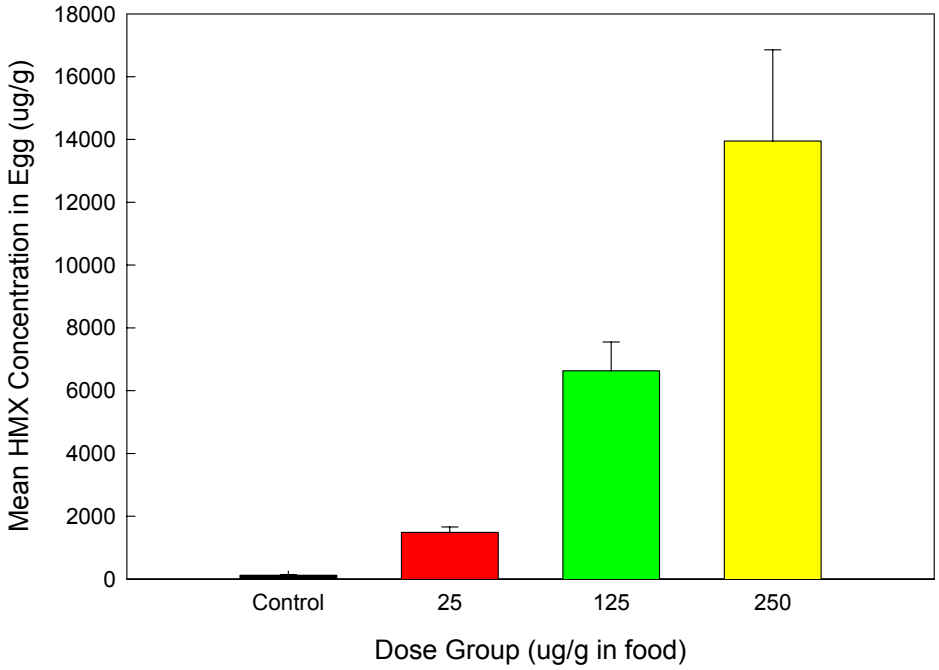


Figure 3. Mean concentrations of HMX in eggs from quail dosed via food. Error bars indicate standard errors.

HMX effects on quail survival and development (2005)

All adult birds survived the entire course of the study; however 1 control pair was removed from the study after the female repeatedly attacked and injured the male, and one high-dose pair was removed 7 weeks after dosing began as the female was injured and ceased laying. No overt signs of toxicity were observed in any adult birds in any dose group. Mean female body weights were greater for birds in the control and low-dose groups than in the medium- and high-dose groups. Similarly, mean male body weights were lower for those the high-dose group compared to the other 3 dose groups. Pairs in the medium- and high-dose groups consumed less food than those in the control and low-dose groups (Table 2).

Table 2. Mean body weight (g) of adult bobwhite quail (*Colinus virginianus*; *n* = 7 pairs), amount of control or HMX-treated poultry feed (g) consumed per pair over 19 3-day feeding periods (20 June - 15 August 2005), and mean egg weights per dose group. The total number of eggs laid per treatment group was 346 in the control, 358 in the low dose group, 285 in the medium dose group, and 283 in the high dose group. Means followed by the same letter were not significantly different at $\alpha = 0.05$.

	Control		Low dose		Medium dose		High dose	
Mass (g)	mean	SE	mean	SE	mean	SE	mean	SE
Females	225.3a	1.8	226.4a	2.2	218.7b	0.9	217.6b	1.7
Males	211.8a	2.2	213.1a	2.6	207.8a	1.3	196.9b	1.7
Food consumed	118.7a	1.4	118.7a	1.2	109.1b	2.0	107.7b	1.3
Eggs	8.7a	<0.1	8.9b	<0.1	8.9b	<0.1	8.7a	<0.1

Mean laying rates (eggs/hen/day) declined after dosing began in all treated groups; however these differences were not statistically significant (Fig. 4) and all females continued to lay at least 1 egg every 1 – 3 days. The mean number of eggs laid per hen during the dosing period was greater in the control (mean = 55) and low dose groups (mean =57) than medium dose (mean =45) and high-dose (mean =44). The mean weight of eggs produced by females in the low- and medium-dose groups was greater than the control and high-dose groups (Table 2).

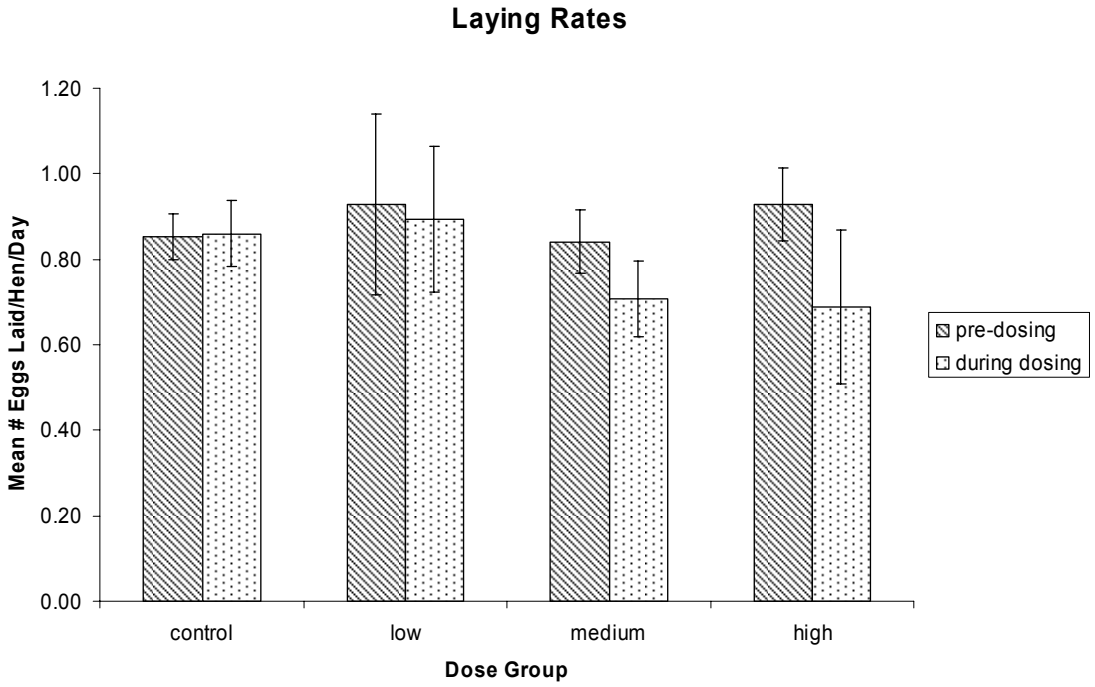


Figure 4. Mean egg-laying rates of adult bobwhite quail before being dosed with HMX (April 2005 – 19 June 2005) and after HMX was added to feed (20 June 2005 – 22 August 2005).

Most eggs died during the 23-day incubation period (79% of eggs set). In all 4 groups, the majority of eggs which died did so during the 1st week of incubation, particularly in the control group. Hatching rate (% of eggs set that hatched) was highest for the medium dose group, followed by the high dose, low dose, and control groups (Table 2). No dose-dependent pattern was observed for any category of final outcome of incubation. The percentage of eggs that died during the 1st week of incubation was highest in the control; the percentage of eggs that died during the 2nd week of incubation was highest for control and low dose; the percentage of eggs that died during the last week of incubation was highest in the high dose group but lowest in the medium dose group; and the percentage of eggs that pipped but died before emerging was highest in the medium dose group (Table 3).

Table 3. Total number and percentage of eggs incubated per treatment group by final incubation outcome (fate). Each group consisted of 7 pairs of adult bobwhite quail (*Colinus virginianus*). Quail in the 3 dose groups were fed HMX-treated poultry feed from 20 June - 22 August 2005.

Fate	Control	Low dose	Medium dose	High dose
Died in first 7 days of incubation	97 (48%)	62 (30%)	46(29%)	47 (27%)
Died during days 8 - 14 of incubation	33 (16%)	33 (16%)	24 (15%)	23 (13%)
Died after day 15 of incubation and before pipping	29 (14%)	44 (21%)	21 (13%)	42 (25%)
Chick began pipping, died before emerging from shell	21 (10%)	32 (15%)	13 (8%)	27 (16%)
Hatched	24 (12%)	38 (18%)	55 (35%)	32 (19%)
Total Eggs Laid By Group	204	209	159	171

HMX transfer to reptile eggs and effects on survival and development (2004)

On average, anoles consumed between 1.5 and 2.5 dosed crickets per week during the study (Fig. 5). There appears to be a trend for lower consumption of dosed crickets in the two HMX groups throughout the study, suggesting an aversion to the HMX.

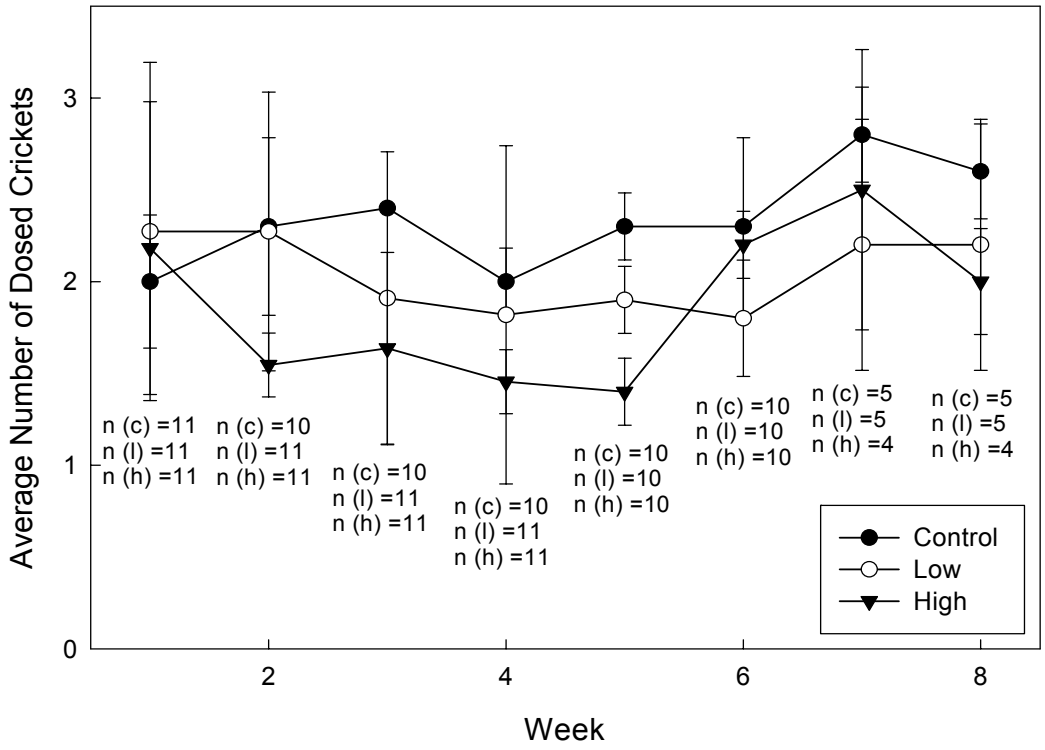


Figure 5. Mean (+SE) number of HMX-loaded crickets consumed per week by anoles in the control (0 HMX), low (500mg/kg HMX), and high (1000mg/kg HMX) dose groups.

Overall consumption of crickets was similar to consumption of dosed crickets, although all groups show a similar pattern during the last half of the exposure period (Fig. 6).

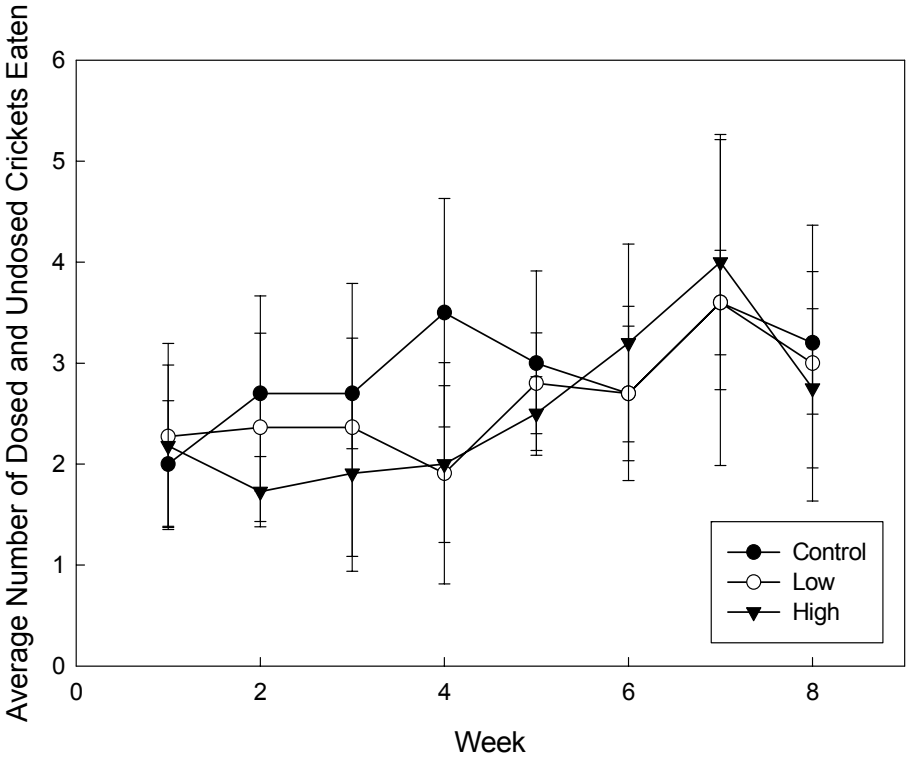


Figure 6. Mean (+SE) number of crickets (with and without HMX) consumed per week by anoles in the control (0 HMX), low (500mg/kg HMX), and high (1000mg/kg HMX) dose groups. See Figure 1 for *n* values.

Body mass declined slightly for anoles in the control and low dose groups, with a more dramatic decline in the high dose group (Fig. 7). On average, body mass of the control anoles at the end of the study was 92% of initial body mass, similar to the low group at 93%. However, at the end of the study, body mass of the anoles in the high group was only 79% of their initial body mass.

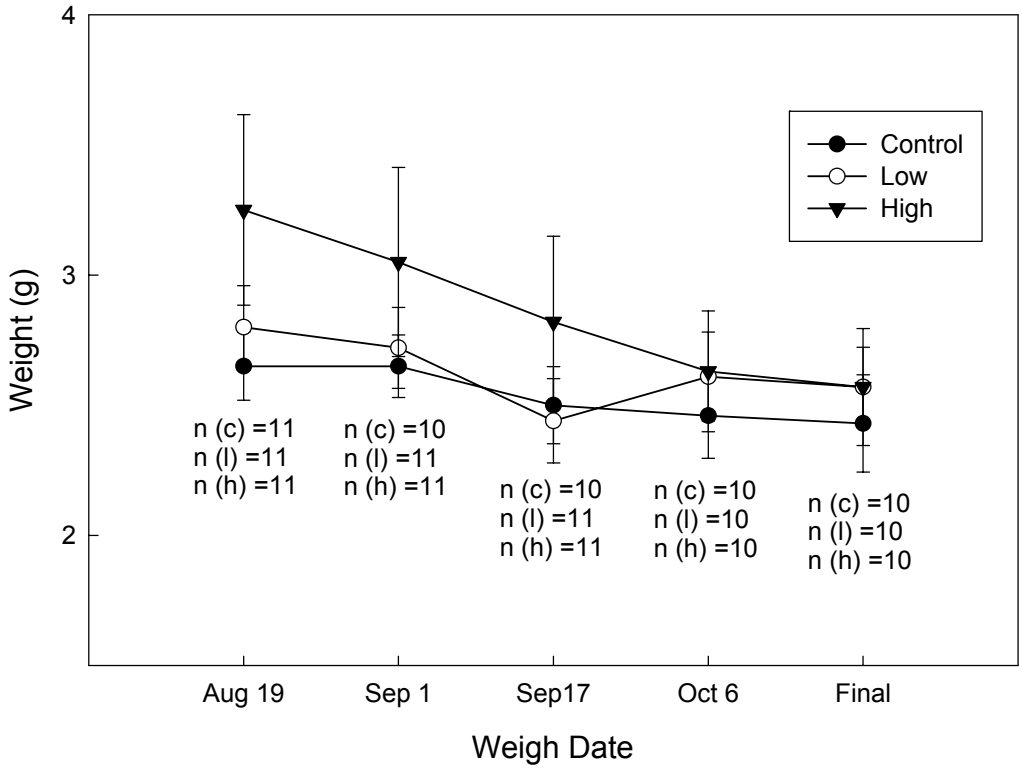


Figure 7. Mean (+SE) body mass of adult female green anoles in the control (0 HMX), low (500mg/kg HMX), and high (1000mg/kg HMX) dose groups.

HMX was detected in eggs from all dose groups, and exhibited a dose-dependant response across groups (Table 4). Concentrations of HMX were variable, particularly in the higher dose groups. Nonetheless, the results indicate that HMX does deposit into eggs in a dose-dependant fashion.

Table 4. Mean HMX concentrations in eggs of green anoles dosed 3-times per week for 8 weeks at 0, 500, and 1000 mg/kg body weight (Control, Low, and High, respectively).

	Control (*n*=2)	Low (*n*=6)	High (*n*=6)
Mean (ng HMX/g sample)	637.41	1953.78	5076.83
SE	48.53	355.61	4105.22

Only five eggs hatched from the 21 eggs incubated. Two eggs were from control animals, with three from high dose animals. All hatchlings survived and had similar growth.

16.0 DISCUSSION

Avian

HMX does not appear to be highly toxic to adult birds. No acute lethality was observed in either quail or red-winged blackbirds dosed at 2,000 mg/kg. However, this study demonstrated that HMX caused sub-lethal effects in adult birds and is readily and very quickly deposited in the eggs of birds exposed via food consumption. Food aversion and weight loss was observed in quail exposed to HMX in food. Therefore, it is unlikely that wild quail (and likely other avian species) would consume food items tainted with significant concentrations of HMX. Since no data on HMX toxicity exists related to birds developing *in ovo*, additional studies are warranted.

Reduced egg production, decreased adult body weights, and decreased food consumption were observed in the medium and high-dose groups. Similar trends have been observed in quail given RDX-treated food (Gogal et al., 2003). Quail fed food treated with the pesticide emamectin benzoate exhibited no apparent effects on body weight and feed consumption. However, higher doses were associated with slightly decreased egg production (O'Grodnick et al., 1998). Overall daily egg production rates (eggs/hen/day) were considerably greater in HMX-treated quail (0.69-0.89) compared to the rates reported for quail treated with emamectin benzoate (0.51-0.57; O'Grodnick et al., 1998). Although they did not report exact rates, Gogal et al. (2003) found that egg production declined in bobwhite quail orally exposed to RDX as dose was increased to ≥ 187 ppm.

In the second phase of the quail study, egg weights and hatching rates did not appear to be correlated to the dose received by the adult birds. This is similar to reproductive results observed in the pesticide-dosed quail (O'Grodnick et al., 1998). Hatching rates in this study (12%-35%) were much lower than those reported by O'Grodnick et al. (58%-70%; 1998) and rates expected in commercial quail operations (75%). As low rates occurred in all groups, and were not dose-dependent, low hatchability was not considered a result of HMX treatment.

The toxicity of HMX is generally considered low in laboratory rodents (Army 1985a, b, c, d). Although HMX can cause weight loss (Phillips et al., 1993) and decreased growth and reproduction in earthworms (Robidoux et al., 2001, 2002), other components of soil in HMX-contaminated areas may confound the effects of HMX. The results of this study on quail further suggest that the toxicity of HMX is relatively low. Furthermore, subchronic impacts on reproduction were also negligible. Additional research involving wild birds inhabiting HMX contaminated areas is needed to determine if the negative impacts of HMX on laboratory quail body condition and subsequent effects on reproductive output occur in natural populations subject to environmental stochastity.

Reptilian

Due to complications in obtaining HMX in a timely manner, the 2004 data are inconclusive as to the effects of HMX on lizard reproductive success. The dosing of adult females was initiated late in the natural breeding season, resulting in low egg production. Thus, any conclusions about HMX effects on egg production, hatching success, or offspring survival and growth are premature. Current studies are in progress and will yield more conclusive information.

The data from 2004 do strongly suggest that HMX deposits in eggs from lizards being repeatedly dosed. The doses used in this study were high, and likely an over-estimate of the actual environmental levels that would be consumed by lizards in the field. In addition, our data

suggest an aversion to HMX, particularly in the highest dose group. These data taken together may indicate that actual dietary exposure could be minimal under normal field conditions.

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A FINAL REPORT

ENTITLED:

PERCHLORATE, THYROID FUNCTION, AND BRAIN DEVELOPMENT IN A GRANIVOROUS PASSERINE

STUDY/PROTOCOL NUMBER: FINCH-04-01

SPONSOR: Strategic Environmental and Research Development Program
SERDP Program Office
901 North Stuart Street, Suite 303
Arlington, VA 22203

TESTING FACILITY:

Name/Address: The Institute of Environmental & Human Health
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University of California: Dr. James R. Millam

RESEARCH INITIATION: September 2003

RESEARCH COMPLETION: December 2004

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GOOD LABORATORIES PRACTICES STATEMENT

Study Number FINCH-04-01 entitled "Perchlorate, thyroid function and brain development in a granivorous passerine", was performed whenever possible in the spirit of the Good Laboratory Practices as outlined in 40 CFR Part 160, August 19, 1989. Noted exceptions are as follows:

Submitted By:

_____ _____

Dr. Michael Hooper
Study Director

Date

QUALITY ASSURANCE STATEMENT

This study was conducted under the Institute of Environmental and Human Health's Quality Assurance Program and whenever possible to meet the spirit of the Good Laboratory Practices as outlined in 40 CFR Part 160, August 19, 1989.

Submitted By:

_____ _____

Ryan Bounds Date
Quality Assurance Manager

1.0 DESCRIPTIVE STUDY TITLE:

Perchlorate, Thyroid Function and Brain Development in a Granivorous Passerine

2.0 STUDY/PROTOCOL NUMBER:

FINCH-04-01

3.0 SPONSOR:

Strategic Environmental and Research Development Program
SERDP Program Office
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4.0 CONTRACT ADMINISTRATOR:

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5.0 TESTING FACILITY NAME & ADDRESS:

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6.0 PROPOSED EXPERIMENTAL START & TERMINATION DATES:

Start Date: September 1, 2003
Termination Date: December 31, 2004

7.0 KEY PERSONNEL:

Dr. Michael J. Hooper, Study Director
Dr. James R. Millam, Test Facility Manager (UC Davis) and co-investigator
Dr. Thomas R. Rainwater, co-investigator
Dr. F. M. Anne McNabb, co-investigator
Mr. Ryan M. Bounds, Quality Assurance Manager
Dr. Ronald J. Kendall, Test Facility Manager (Texas Tech)

8.0 STUDY OBJECTIVES /TEST SYSTEM JUSTIFICATION:

Objective

The objective of this research was to evaluate the responsiveness of zebra finches (Taeniopygia guttata) to perchlorate exposure by:

- Orally dosing nestlings with perchlorate on post-hatch days (PHD) 3-14
- Monitoring nestling behavior and growth during the dosing period
- Monitoring post-fledging behavior (timing of shift from foraging to fledging)
- Euthanizing dosed birds on PHD 15, 40, and 72 for tissue collection
- Measuring thyroid hormone concentrations in bird plasma and thyroid glands
- Examining thyroid histology of dosed birds
- Examining brain histology and morphometry of dosed birds
- Examining perchlorate accumulation in the liver of dosed birds

Purpose

The primary purpose of this research is to determine if exposure to perchlorate results in alterations in thyroid hormone levels and subsequent adverse effects on development and behavior in a granivorus bird.

Perchlorate is an environmental contaminant known to impair thyroid function in mammals (Saito et al., 1983; Thuett et al., 2000a,b), and recent studies indicate that perchlorate may also disrupt normal thyroid function in birds (McNabb, 2002a,b, 2004a,b; Smith et al., 2003a,b). In this study, we tested the hypothesis that perchlorate exposure in nestling birds leads to thyroid disturbances that influence brain development whose repercussions can be measured using growth, behavior, and histopathology.

The United States Environmental Protection Agency has noted a need for the determination of the effects of dietary exposure to perchlorate in birds. Dr. Anne McNabb of Virginia Tech has noted perchlorate-induced alterations in circulating thyroid hormones of adult and young northern bobwhite quail (McNabb et al., 2004a,b). Perchlorate has been detected by Texas Tech University researchers at high concentrations in water and plant vascular tissues and seeds at concentrations ranging from approximately 1 ppm to as high as 5000 ppm (Smith et al. 2001). Passerine birds living in contaminated environments have also been shown to contain perchlorate (McMurry et al., 2003) and may be at risk of disruption of normal thyroid function and subsequent alterations in reproduction, growth, and behavior. In the present study, the zebra finch was used as a model for assessing the effects of perchlorate exposure on normal thyroid function and related endpoints in birds.

Justification of test system

The following items outline support of perchlorate as an environmental contaminant of concern and the zebra finch as an appropriate test system.

- Deer mice (Peromyscus maniculatus) exposed to perchlorate in the laboratory exhibit altered thyroid hormone concentrations and reduced numbers of active thyroid follicles (Thuett et al., 2000a,b)
- Developing and adult bobwhite quail (Colinus virginianus) exposed to perchlorate in the laboratory exhibit alterations in thyroid gland hormone content, thyroid gland weight, and plasma thyroid hormone concentrations (McNabb, 2002a,b; McNabb, 2004a,b)
- Adult quail and mallards (Anas platyrhynchos) exposed to perchlorate in the laboratory exhibit thyroid gland hyperplasia (Smith et al., 2003b)
- Exposure of adult quail to perchlorate results in maternal transfer and accumulation of perchlorate in eggs (Smith et al., 2003a)
- Perchlorate has been detected in bodily tissues from various wild passerines and in wood duck (Aix sponsa) eggs collected from perchlorate-contaminated sites, demonstrating that perchlorate is capable of accumulating in birds and eggs under environmentally relevant exposure scenarios
- Oral exposure to other endocrine disrupters, environmental estrogens, induces changes in zebra finch chick brain development (Quaglino et al., 2002), and such exposure correlates with subsequent adult behavioral deficits (Gurney and Konishi, 1980; Gurney, 1982)
- Due to its attributes as a laboratory test species as well as its environmental relevance as a granivorous passerine, the zebra finch is an excellent model for assessing the effects of perchlorate exposure on normal thyroid function and related endpoints in this avian group

In this study, zebra finches were monitored in a proven captive aviary scenario where adult birds were allowed to nest and raise young in provided nest boxes. This species was chosen due to its proven success in captivity, its obligate granivory (a relevant perchlorate exposure pathway), and previous research data sets on its brain structure and neuronal architecture (Quaglino et al., 2002). Research on the dimorphic song control nuclei of the zebra finch brain has led to substantial knowledge of normal development and thus provides a model with which we can observe perturbations induced by perchlorate.

9.0 TEST ANIMALS (number, weight, source, strain):

Species: Zebra finch (Taeniopygia guttata)

Strain: local avicultural stock

Age: hatching – 72 days

Number: 192

Source: Institutionally bred in colony reproduction facilities at the University of California, Davis, CA

10.0 METHODS:

Husbandry and identification of test species

Adult zebra finches bred independently in an established colony (UC Davis) and used provided nest boxes to build nests, lay eggs, and hatch nestlings. Each breeding pair was housed in a separate standard bird cage (46 X 46 X 41 cm) labeled with a note card containing the appropriate identification information for the animals on the front of the cage. Adult birds were leg-banded with a uniquely-numbered leg band. Each egg laid in a given nest was uniquely numbered using a sharpie and data on each egg (e.g., oviposition date, hatch date, etc.) was recorded on a second cage card attached to the nest box. Each nestling in a clutch was identified by dying down feathers with red, blue, green, or yellow food coloring. If necessary, one nestling remained uncolored ("natural") to differentiate it from its colored siblings. Just prior to or immediately following fledging, nestlings were banded with a uniquely-numbered leg band. Collected samples were placed in individually labeled bags/containers and stored appropriately according to TIEHH SOP IN-3-02.

Nestling dosing

On PHD 3-14, nestling finches were orally dosed with 1 µl / g body mass of one of the following solutions: 0 (control; nano-pure water vehicle), 0.01, 0.1, or 1.0 g / ml sodium perchlorate (0, 10, 100, and 1000 µg / g body mass doses, respectively). Perchlorate dosing solutions were validated analytically prior to their use. Nestling begging behavior facilitates the dosing process, and doses were delivered by expressing the dose into the upwardly turned mouths of the nestlings using a 20 µl pipette. Approximately 16 finches were treated at one of the four dose concentrations to yield approximately six to eight males and females per dose group. The breakdown of dose groups is as follows.

There were 12 dose x age study groups:

Four dose levels: 0, 10, 100, 1000 µg perchlorate / g body weight

Three nestling ages: 15, 40 and 72 days

12 groups at 16 nestlings per group = 192 nestlings. No two nestlings from a single nest were assigned the same dose/age combination.

Nestling growth

Zebra finch growth was measured by examining two indices, body weight and tibiotarsal length. Just prior to dosing, each bird was weighed using an electronic balance (nearest

0.01 g) and the length of its right tibiotarsus (long leg bone between the femur and tarsometatarsus) measured with calipers (nearest 0.1 mm). In addition, brain mass (72 day-old males only) and liver mass (15 and 40 day-old males and females, 72 day-old females) were recorded (wet weight) immediately following collection of these tissues from necropsied birds.

Nestling termination and tissue collections

Nestlings were euthanized on PHD 15 (at fledging), PHD 40 (when adult dependence ceases [Zann, 1996]), and PHD 72 (the time at which they reach sexual maturity). At each of the collection time points, birds were euthanized with an IP injection of equithesin [chloral hydrate:sodium pentobarbital (4:1)]. All birds collected on PHD 15 and PHD 40 were immediately necropsied and blood, liver and thyroid collected.

Zebra finches exhibit extreme sexual dimorphism of the brain; males have four highly developed song control nuclei, while in females these nuclei are greatly reduced or non-detectable (Quaglino et al., 2002). The presence and size of these song control nuclei correlate with singing behavior such that in zebra finches, males sing but females do not (Nottebohm and Arnold, 1976; Quaglino et al., 2002). Thus, we examined male brains for potential perchlorate-induced alterations of the song control nucleus area X of the paraolfactory lobe.

PHD 72 day-old males were deeply anesthetized with sodium pentobarbital and perfused with 0.1 M sodium phosphate buffered saline (pH 7.4) followed by 2% paraformaldehyde in 0.1 M sodium phosphate (pH 7.4) solution containing 0.03 % glutaraldehyde for 20 min (Quaglino et al., 2002). Brains were removed, weighed (ww) and preserved until sectioning.

To avoid contamination of tissues by perfusate, PHD 72 day-old females were not perfused and instead necrospied immediately following euthanasia for collection of blood, liver and thyroid. In additon, right thyroid glands of males fixed as a result of the perfusion were also collected for histopathology.

Brain morphometry

Brains were sliced into 50-µm sections, mounted on slides, Nissl-stained using thionin, and coverslipped for viewing (Quaglino et al., 2002). Area X was outlined and its nuclear volume calculated after digital reconstruction of serial sections using Neurolucida morphometric software (Quaglino et al., 2002).

Histopathology

Thyroids were collected and placed in buffered formalin for histopathological sectioning and morphological assessment. Prior to processing, the collection of fixed thyroids was inadvertently frozen. Groups of samples were subsequently processed, sectioned and stained and compared with thyroids from birds that were not frozen prior to preparation.

Unfortunately, the freezing and thawing of the samples resulted in artifacts in the tissues that percluded measurement of colloid and cell heights for histological comparison between the birds at different dose levels. No further quantitative work was performed with these samples

Hormone analyses

Thyroid hormone concentrations in chick blood plasma and thyroid gland were determined by radioimmunoassay (RIA) (McNabb and Cheng 1985; Wilson and McNabb, 1997; McNabb et al., 2004b) optimized with pooled zebra finch samples.

Analytical chemistry

Perchlorate accumulation in liver was determined using an extraction method optimized for animal tissue (TIEHH SOP GW-02-03) and analyzed using ion chromatography (Anderson and Wu, 2002). Because of the small size of zebra finch livers, samples from all 40 and 72 day birds and the 15-day 10 ug/g birds were combined into sex-specific composites to improve detection limits.

Behavior

Effects of perchlorate exposure on nestling behavior and growth were examined as follows.

General composure – Within a clutch, the behavior of the nestling receiving the control dose (water) was considered normal. Any behavior by dosed siblings that deviated from the normal behavior exhibited by the control chick was noted. These observations of general composure were made incidentally as researchers were checking nest boxes, marking and dosing chicks, or engaging in routine bird maintenance. If any unusual behavior was noticed, as compared to the behavior of the control sibling in that clutch, it was recorded.

Begging frequency and intensity – Begging frequency and intensity was assessed by monitoring the crop status and begging behavior of each chick just prior to dosing.

Crop status - Crop status was assessed based on the assumption that a positive relationship exists between frequency and intensity of begging (in the nest) and the amount of food in the crop. On PHD 10-14, before dosing, crop status and begging frequency/intensity was scored for each bird. Crop status scores were assessed by visual inspection of each bird's crop region and scored as 0 (empty; no seed visible through skin), 1 (moderately full; at least one seed visible and skin around crop is loose or slightly distended), or 2 (full; crop is packed with seed such that skin around crop is distended on both sides of neck and stretched tightly, and much seed is visible).

Begging – Begging intensity was further assessed by monitoring the begging behavior of each bird on PHD 10-14. Just prior to dosing, each chick was placed in an artificial nest cup lined with a Kim-wipe®. The appropriate dose for a given bird was drawn up into a pipette tip, and the pipette tip was then slowly moved toward the chick's bill from the front-facing direction. Begging intensity was scored based on each chick's response to the pipette tip as 0 (bird does not beg, must have mouth forced open to administer dose), 1 (bird opens mouth and begs after two taps of bill with pipette tip), 2 (bird opens mouth and begs after one tap of bill with pipette tip), and 3 (bird opens mouth and begs with no taps of bill with pipette tip).

Open field test – Bird behavior was monitored in an open field test just prior to dosing on PHD 10-19. The test arena was a 46 X 46 X 41 cm wire mesh breeding cage (Quaglino et al., 2002). A 21.6 X 28 cm (8.5 X 11inch) sheet of paper with a circle (diameter = 11.8 cm) drawn in the center was placed on the center of the cage floor. Each chick was placed in the center of the circle facing the same direction (left of the cage door), the cage door closed, and the bird's behavior observed for 30 seconds. Observers recorded whether or not the bird left the circle and whether or not it attempted to fly. In addition, the bird's location in the cage at the end of the 30 second period was noted. Cage locations were identified as one of the following:

1 = in circle
2 = on floor in back left corner (left of cage door)
3 = on floor along back wall of cage, between the two corners
4 = on floor in back right corner
5 = on floor along right wall, between back and front corners
6 = on floor in front right corner
7 = on floor along front wall of cage between two front corners (just below cage door)
8 = on floor in front left corner
9 = on floor along left wall, between back and front corners
10 = on wooden perch, above cage floor
11 = perched on cage wall, above cage floor

Weaning assay – To examine potential perchlorate-induced alterations in the timing of the shift from begging to foraging (weaning), birds in the PHD 40 and 72 test groups were tested as follows. On the morning of PHD 28, each bird was removed from its home cage, weighed, and placed into a separate cage (same size as home cage; 46 X 46 X 41 cm) (henceforth referred to as "weaning cage"), alone, for approximately 8 hr. During

this time, each bird had access to food and water. At the end of the 8 hr period, the bird was removed from the weaning cage, weighed again, and placed back into its home cage. The bird's behavior was then monitored for 5 minutes. Observers recorded whether the bird begged for food from its parents or whether it fed itself. If the bird begged for food, it was assumed that it did not feed, at least adequately, during its 8 hr alone in the weaning cage, and the bird was considered unweaned. If the bird fed itself or did not beg, the bird was considered weaned.

Prior to official use in the study, this weaning assay was validated by testing un-dosed birds of various ages (PHD 20 25, 30, 35, 40) to determine the approximate time of weaning. It was determined that most birds at PHD 25 or younger begged when placed back into their home cages after 8 hr alone, while most birds at PHD 30 or older did not beg and often fed themselves when returned to their home cages. Thus, PHD 28 was selected as the "threshold day" on which we suspected the shift from begging to foraging to occur.

Statistical analyses

Statistical differences in histological measures, hormone concentrations, song nuclear volume, brain mass, and liver mass between and among dose groups were examined using one-way ANOVA. Differences in body mass and tibiotarsal length among dose groups over time were examined using repeated measures ANOVA (linear fixed effects model). Differences in behavioral indices were examined using two-way ANOVA. Data violating assumptions of parametric analysis (prior to or following transformation) were examined using appropriate non-parametric substitutes. Differences were considered significant when $p \leq 0.05$.

11.0 RESULTS

Growth

Body mass. Chick body mass was significantly reduced in the 100 µg/g ($p = 0.0538$, 2% decrease) and 1000 µg/g ($p < 0.0001$, 7% decrease) dose groups from PHDs 3-14 (Table 1, Figure 1). This reduction in body mass was also observed on PHD 40 ($p = 0.0286$) but not PHD 72.

Tibiotarsal length. Chick tibiotarsal length was significantly reduced in the 100 µg/g ($p = 0.0056$, 5% decrease) and 1000 µg/g ($p < 0.0001$, 9% decrease) dose groups from PHDs 3-14 (Table 2, Figure 2).

Brain mass. In 72 day-old birds, mean brain mass in the 100 µg/g and 1000 µg/g dose groups was significantly greater (6% and 8%, respectively; $p = 0.0517$) than in the control group (Table 3, Figure 3).

Liver mass. In 15 day-old birds, liver mass in the 100 µg/g and 1000 µg/g dose groups was significantly greater (24% and 27%, respectively; p = 0.0007) than in the control group (Table 4, Figure 4). In 40 and 72 day-old birds, liver mass did not differ significantly among dose groups (Table 4, Figure 4).

Brain morphometry

Mean nuclear volume of area X in brains of 72 day-old male zebra finches did not differ significantly among dose groups (Table 5, Figure 5).

Hormone analysis

Thyroid T4 levels did not follow particular age- or dose- dependent patterns when measured on days 15, 40 and 72 (Figure 6). Plasma T4 concentrations in post-hatch day 15 birds started at higher levels and decreased with age, and were decreased at higher doses on day 15, though on days 40 and 72, there were no apparent differences between controls and treatment birds (Figure 7). Plasma T3 levels were also higher in the nestlings, decreased in post-hatch day 40 birds and rose slightly by day 72.

Analytical chemistry

Perchlorate levels increased in a dose-dependent manner in post-hatch day 15 nestlings, rising up to nearly 10 ug/g in the highest dosed nestlings (Table 6). There was high variability in the concentrations, with standard deviations exceeding the mean in several dose/sex groups. Perchlorate concentrations in 40 and 72 day birds were either not detectable or occurred at low levels in a fraction of the samples. Low level concentrations were detected in two of the samples from control birds, though the detected levels fell below the general limits of detection. As the data represent a mixture of analyses using individuals and composites, results were assessed on a qualitative rather than quantitative basis.

Behavior

Begging frequency and intensity. Both of these endpoints were affected by perchlorate exposure at the highest dosing levels. *Begging activity* was significantly influenced by perchlorate dosing at the 100 and 1000 ug/g dose levels (Table 7, Figure 8). On post-hatch days 11 through 14, 1000 ug/g chicks had higher begging scores than the 0 and 10 ug/g groups. The 100 ug/g chicks begged more than controls on days 12 and 13 and more than the 10 ug/g group on day 13. *Crop status* was also influenced by dose, with 1000 ug/g chicks having higher crop scores than both the control and the 10 ug/g groups on day 14 (Table 8, Figure 9).

Open field test. Perchlorate exposure to zebra finch nestling influenced open field behavior both in the chicks' willingness to venture beyond their initial physical location as well as their attempts to fly. *Movement* outside of the central circle by 1000 ug/g chicks differed by varying degrees compared to all other dosing groups (Tables 9 and 11,

Figure 10). The 1000 ug/g group was decreased compared to controls on post-hatch days 10 through 18, compared to the 10 ug/g chicks on days 10, 11, 13, 14, 16 and 17, and compared to the 100 ug/g chicks on day 16. The other dosed groups did not differ significantly from controls or each other. *Flying attempts* first occurred on post-hatch day 14, and immediately, the 1000 ug/g group made significantly less attempts to fly compared to controls, a pattern that continued through day 19 (Table 10 and 11, Figure 10). On days 16 through 19, flight attempts in the 1000 ug/g group were also less than those of all other dosing groups. Of the other dosing groups, the 100 ug/g group made fewer flight attempts compared to controls on post-hatch days 15 and 17, while flight attempts in the 10 ug/g group were decreased only on post-hatch day 15.

Weaning assessment. When confronted with a parent-free environment for 8 hrs, PHD 28 chicks from the highest dose group were least capable of feeding themselves and maintaining body weight. *Begging activity*, once returned to the parents, was significantly greater in the 1000 ug/g chicks than those from the control and 10 ug/g dose groups (Tables 12 and 14, Figure 11). *Loss of body mass* was significantly greater in the 1000 ug/g chicks than in chicks from the control, 10 and 100 ug/g dosing groups (Table 13 and 14, Figure 11). When compared to controls, the 1000 ug/g birds lost twice as much weight during the 8 hour test period.

12.0 DISCUSSION

Perchlorate dosing of zebra finch nestlings from PHD 3 to 14 led to a dose-dependent increase of circulating perchlorate that was reflected in liver perchlorate levels in 15 day nestlings. By days 40 and 72, the majority of samples lacked the highly elevated levels of perchlorate seen in the PHD 15 nestlings. This accumulation pattern occurred without substantial bias toward either sex.

Growth assessments indicated that perchlorate exerted an immediate inhibitory effect on body mass and tibio-tarsal length at the 100 and 1000 ug/g dose groups, while concurrently increasing PHD 15 liver mass in the same two groups. Despite the substantially lower perchlorate levels (as indicated in liver residues) in chicks collected on PHD 40 and 72, longer-term changes were seen in a continued decreased body mass through PHD 40 and an increase in PHD 72 brain mass, both occurring in the 100 and 1000 ug/g dose groups. Despite the increased brain size seen in the PHD 72 male chicks at higher doses, the nuclear volume of area X did not change significantly. These data provide a strong indication that perchlorate influences nestling growth in a dose-dependent manner, with immediate and long-term effects reflected in body and tissue mass and bone length at the 100 and 1000 ug/g dose levels.

Neither thyroid T4 nor plasma T4 or T3 levels responded in a consistent dose- or age-dependent manner. As this is the first attempt to assess these endpoints, more detailed examination of normal developmental patterns of these hormones, beyond the 15, 40 and 72 day endpoints, would benefit the interpretation of these data.

Behavioral assessments provided data on nestling hunger and responsiveness to parents, motivation for spontaneous behavior, and capacity for weaning from a begging nestling to a foraging juvenile finch. Nestlings in both the 100 and 1000 ug/g dose groups had greater begging scores than controls and the 10 ug/g groups, with greater significance in the 1000 ug/g group, reflected in the significantly greater crop status values in this highest dose. Interestingly, these two groups with more aggressive begging and greater crop contents were the same two groups with reduced body mass and bone length during the nestling stage of development, suggesting that perchlorate's effects on food seeking behavior may be associated with its reduction in physical growth capacity in the 100 and 1000 ug/g dose groups.

The open field test provided the greatest responsiveness to perchlorate exposure of any endpoint. Movement out of the test circle was significantly depressed in the highest dose group throughout the PHD 10 to 19 period. The influence on attempts to fly was more dramatic, with all perchlorate-dosed bird groups demonstrating significantly lower proportions of chicks attempting to fly on PHD 15 and none of the dosed groups reaching even half of the proportion that attempted flight in the controls. The 1000 ug/g dose group was particularly affected in its attempts at flight. When 63, 66 and 70 percent of the PHD 17, 18 and 19 control birds, respectively, were attempting to fly, only 2, 8 and 22 percent of the same aged birds in the high dose group made such an attempt. In the wild, such a depression in flight initiation might affect a nestling's ability to effectively fledge the nest or increase the residence time of the nestling in the nest.

A decrease in the ability of zebra finch chicks to wean themselves from their parents' care was seen at the highest dose level where the 1000 ug/g chicks demonstrated increased begging activity and a greater loss of body mass when separated from their parents for an 8 hr period. As was seen in the open field test, perchlorate exposure at the highest level appears to delay the time at which the chick can effectively be weaned from parental care and move toward independence in the field.

Overall, the findings of this study demonstrate that perchlorate exposure in zebra finches led to a dose-dependent impediment of a variety of growth and behavioral endpoints. Effects on growth occurred at both the nestling as well as sub-adult and adult stages. Behavioral effects were of a type that, should they occur in the wild, could lead to delays in fledging or inability to fledge from the nest and delays in weaning or inability to wean from adult care. Though these latter effects were seen most extensively in the highest dose group, they occurred sporadically and in a significant dose-dependent manner at lower doses. As the dose levels in this study fall within those predicted for granivorous birds inhabiting perchlorate contaminated environments (McNabb et al. in press) it is reasonable to conclude that perchlorate exposure at those sites may be adversely affecting reproductive success of obligate avian granivores.

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14.0 ACKNOWLEDGMENTS

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Table 1. Mean (± SE) body mass (g) of nestling zebra finch chicks dosed with sodium perchlorate on post-hatch days (PHD) 3 to 14. Number of chicks measured on a given PHD for a given dose ranged from 70 to 86 chicks.

Dose (µg/g)	Body mass (g) on post-hatch days 3-14											
	3	4	5	6	7	8	9	10	11	12	13	14
0 (control)	2.3 ± 0.1	3.0 ± 0.1	3.9 ± 0.1	4.8 ± 0.2	5.9 ± 0.2	6.9 ± 0.2	8.0 ± 0.2	8.7 ± 0.2	9.4 ± 0.2	9.9 ± 0.2	10.1 ± 0.2	10.2 ± 0.1
10	2.2 ± 0.1	2.9 ± 0.1	3.8 ± 0.1	4.8 ± 0.1	5.8 ± 0.1	6.7 ± 0.2	7.8 ± 0.2	8.4 ± 0.2	9.2 ± 0.2	9.9 ± 0.2	10.2 ± 0.1	10.3 ± 0.1
100	2.3 ± 0.1	2.9 ± 0.1	3.8 ± 0.1	4.6 ± 0.2	5.4 ± 0.2	6.3 ± 0.2	7.1 ± 0.2	7.9 ± 0.2	8.5 ± 0.2	9.2 ± 0.2	9.6 ± 0.2	10.0 ± 0.2
1000	2.4 ± 0.1	3.1 ± 0.1	3.8 ± 0.1	4.6 ± 0.1	5.4 ± 0.2	6.3 ± 0.2	7.0 ± 0.2	7.6 ± 0.2	8.2 ± 0.2	8.3 ± 0.2	9.0 ± 0.2	9.6 ± 0.2

Table 2. Mean (± SE) tibiotarsal length (mm) of nestling zebra finch chicks dosed with sodium perchlorate on post-hatch days (PHD) 3 to 14. Number of chicks measured on a given PHD for a given dose ranged from 72 to 86 chicks.

Dose (µg/g)	Tibiotarsal length (mm) on post-hatch days 3-14											
	3	4	5	6	7	8	9	10	11	12	13	14
0 (control)	10.2 ± 0.1	11.4 ± 0.1	12.8 ± 0.2	14.2 ± 0.2	15.6 ± 0.2	17.2 ± 0.2	18.9 ± 0.2	20.1 ± 0.2	21.1 ± 0.2	21.8 ± 0.2	22.3 ± 0.2	22.8 ± 0.2
10	9.9 ± 0.1	11.1 ± 0.2	12.6 ± 0.2	14.1 ± 0.2	15.8 ± 0.2	17.3 ± 0.2	18.7 ± 0.2	19.8 ± 0.2	20.8 ± 0.2	21.8 ± 0.2	22.5 ± 0.1	22.9 ± 0.1
100	10.0 ±	11.3 ±	12.5 ±	13.8 ±	15.0 ±	16.3 ±	17.4 ±	18.4 ±	19.4 ±	20.3 ±	21.1 ±	21.6 ±

	0.1	0.2	0.2	0.2	0.2	0.3	0.3	0.3	0.3	0.3	0.3	0.3
1000	10.0 ± 0.1	11.4 ± 0.1	12.8± 0.2	14.0 ± 0.2	15.1 ± 0.2	16.0 ± 0.2	17.0 ± 0.2	18.0 ± 0.3	18.8 ± 0.2	19.4 ± 0.3	20.1 ± 0.2	20.6 ± 0.2

Table 3. Mean (± SE) brain mass (g) of 72 day-old male
zebra finches dosed with sodium perchlorate on post-hatch days 3 to 14.

Dose (µg/g)	n	Brain mass (g)
0 (control)	14	0.43 ± 0.01
10	12	0.44 ± 0.01
100	12	0.46 ± 0.01
1000	10	0.47 ± 0.01

Table 4. Mean (± SE) liver mass (g) of zebra finches dosed with sodium perchlorate on
post-hatch days (PHD) 3 to 14. Numbers in parentheses indicate numbers of livers weighed.

	Liver mass (g)		
Dose (µg/g)	PHD 15	PHD 40	PHD 72*
0 (control)	0.30 ± 0.01 (19)	0.37 ± 0.01 (23)	0.48 ± 0.04 (11)
10	0.29 ± 0.01 (22)	0.35 ± 0.02 (20)	0.48 ± 0.07 (12)
100	0.37 ± 0.02 (19)	0.38 ± 0.02 (22)	0.45 ± 0.03 (16)
1000	0.38 ± 0.03 (9)	0.37 ± 0.02 (18)	0.41 ± 0.02 (13)

*Females only

Table 5. Mean (± SE) volume (mm^3) the song control nucleus area X in brains of 72 day-old male zebra finches dosed with sodium perchlorate on post-hatch days 3 to 14.

Dose (µg/g)	n	Nuclear volume (mm^3)	Range
0 (control)	6	1.35 ± 0.06	1.14 – 1.52

10	5	1.39 ± 0.12	1.08 – 1.83
100	7	1.17 ± 0.17	0.67 – 2.03
1000	5	1.28 ± 0.14	0.97 – 1.72

Table 6. Perchlorate concentration in livers from zebra finches dosed with sodium perchlorate on post-hatch days 3 to 14.

		Perchlorate concentration (ng/g) by days post-hatch and sex of zebra finches					
		15		40		72	
Dose		M	F	M	F	M	F
0	Mean or range	nd	nd	122	nd	nd	126
	SD	--	--	40.1	--	--	33.3
	N	11	10	1,C13	1, C7	C2	1, C12
	n	0	0	1	0	0	1
10	Mean or range	421	279	1166	nd	na	843
	SD	318	270	1437	--	--	980
	N	2,C3,C4	C3,C3,C3,C4	1,C6	1,C5,C6	0	1,C10
	n	2	3	1	0	0	1
100	Mean or range	nd - 1415	722	nd	nd - 94.9	na	nd
	SD	--	724	--	--	--	--
	N	9	10	1,C4,C5	1,C6,C7	0	1,C7,C7
	n	3	6	0	1	0	0
1000	Mean or range	4513	9450	177	nd	na	nd - 115
	SD	4686	19738	88	--	--	--
	N	4	7	1,C5,C4	1,C4,C4	0	1,C6,C6,C5
	n	3	5	2	0	0	1

N is the total number of samples and composites analyzed. Composites are denoted C*X*, with *X* indicating the number of samples in the composite. n is the number of samples or composites in which perchlorate was detected. Means were determined when n $\geq$ 50% of N. Limit of detection was 300 ng/g. One-half the detection limit, 150 ng/g, was assigned to samples with non-detectable perchlorate levels when means were determined. nd

indicates not detectable. na indicates not analyzed. -- indicates measure cannot be made.

Table 7. Begging behavior in nestling zebra finches (post-hatch ages 10 to 14 days) dosed with perchlorate on post-hatch days 3 to 14.

Perchlorate Dose (ug/g)	Age (days post hatch)				
	10	11	12	13	14
0 (control)	1.21	1.16	0.82	0.65	0.51
10	1.61	1.15	0.96	0.54	0.52
100	1.43	1.57	1.37	1.14	0.88
1000	1.47	1.63	1.55	1.19	1.17

Table 8. Crop scores of nestling zebra finches (post-hatch ages 10 to 14 days) dosed with perchlorate on post-hatch days 3 to 14.

Perchlorate Dose (ug/g)	Age (days post-hatch)				
	10	11	12	13	14
0 (control)	1.55	1.53	1.32	1.23	1.07
10	1.46	1.59	1.43	1.24	1.05
100	1.69	1.41	1.40	1.24	1.19
1000	1.58	1.58	1.45	1.40	1.40

Table 9. Comparative open field mobility of nestling zebra finches (post-hatch ages 10 to 19 days) dosed with perchlorate on post-hatch days 3 to 14: Percentage of nestlings moving beyond test limits in open field test cage.

Age (days post-hatch)	Perchlorate dose (ug/g body wt)			
	0	10	100	1000
10	44.2	36.1	21.6	13.9
11	53.9	48.6	27.6	19.2
12	48.6	42.5	36.8	22.2
13	47.4	50.7	35.1	24.6
14	57.9	60.8	37.3	30.0
15	66.7	54.2	49.0	32.7
16	72.9	63.0	58.0	31.5
17	79.2	73.9	66.7	49.1
18	87.2	80.0	72.5	64.0

19	87.0	82.2	85.4	70.0

Table 10. Comparative open field mobility of nestling zebra finches (post-hatch ages 10 to 19 days) dosed with perchlorate on post-hatch days 3 to 14: Percent of nestlings that attempted to fly.

	Perchlorate dose (ug/g bw)			
Age	0	10	100	1000
10	0.00	1.39	0.00	0.00
11	1.32	1.35	0.00	0.00
12	1.39	1.37	1.32	1.39
13	2.63	2.74	0.00	0.00
14	9.21	1.35	4.00	0.00
15	27.1	10.4	4.08	0.00
16	35.4	28.3	20.0	0.00
17	62.5	45.7	33.3	1.89
18	66.0	64.4	49.0	8.00
19	69.6	68.9	58.3	22.0

Table 11. Statistical analysis of zebra finch nestling open field test endpoints as a function of nestling age and perchlorate dose level.

	Significant differences (P<0.05)	
Variable	Age (days)	Dose groups (ug/g mass)
Leave circle	10-18	1000 < 0
Leave circle	10, 11, 13, 14, 16, 17	1000 < 10
Leave circle	16	1000 < 100
Fly	14-19	1000 < 0
Fly	16-19	1000 < 100, 10, 0
Fly	15	1000, 100, 10 < 0
Fly	17	100 < 0

Table 12. Begging behavior of perchlorate-dosed zebra finch chicks following 8-hr isolation from parents. Begging behavior was quantified for first 5 minutes with parents after 8 hr isolation.

Perchlorate Dose (ug/g)	N	Mean	SE
0 (control)	43	1.23	0.07
10	43	1.33	0.07
100	40	1.38	0.08
1000	32	1.68	0.08

Table 13. Mass loss of perchlorate-dosed zebra finch chicks following 8-hr isolation from parents. Body mass change (lower graph) occurred over the 8 hr isolation period.

Perchlorate Dose (ug/g)	percent mass change	Abs SE
0 (control)	-4.56	0.49
10	-5.25	0.54
100	-6.64	0.77
1000	-9.71	1.00

Table 14. Statistical analysis of zebra finch nestling weaning assay endpoints as a function of perchlorate dose level.

Variable	Significant differences (P<0.05) between dose groups (ug/g mass)
Begging behavior	1000 > 10, 0
Body mass loss	1000 > 100, 10, 0

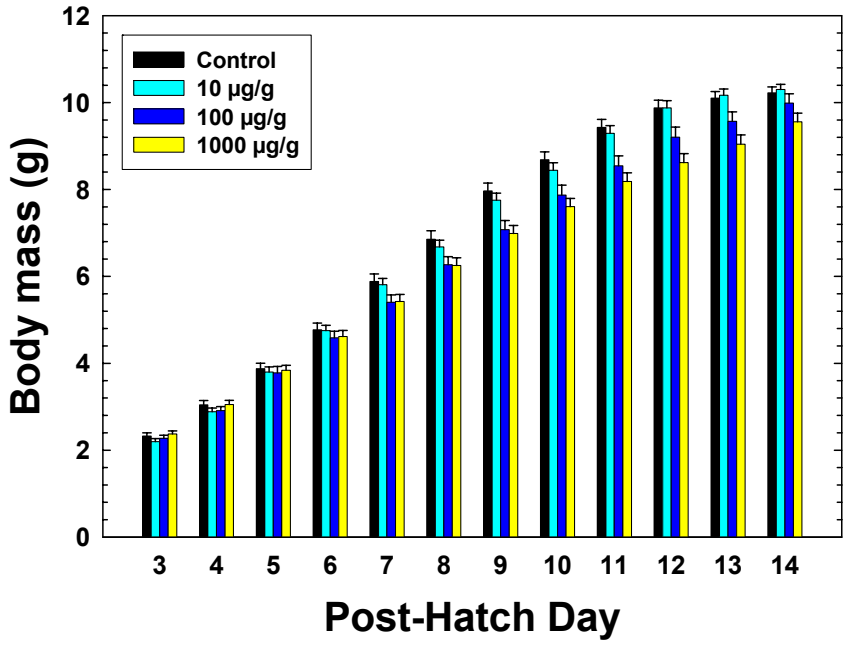


Figure 1. Mean (±SE) body mass (g) of zebra finch chicks dosed with sodium perchlorate once per day on post-hatch days 3 to14.

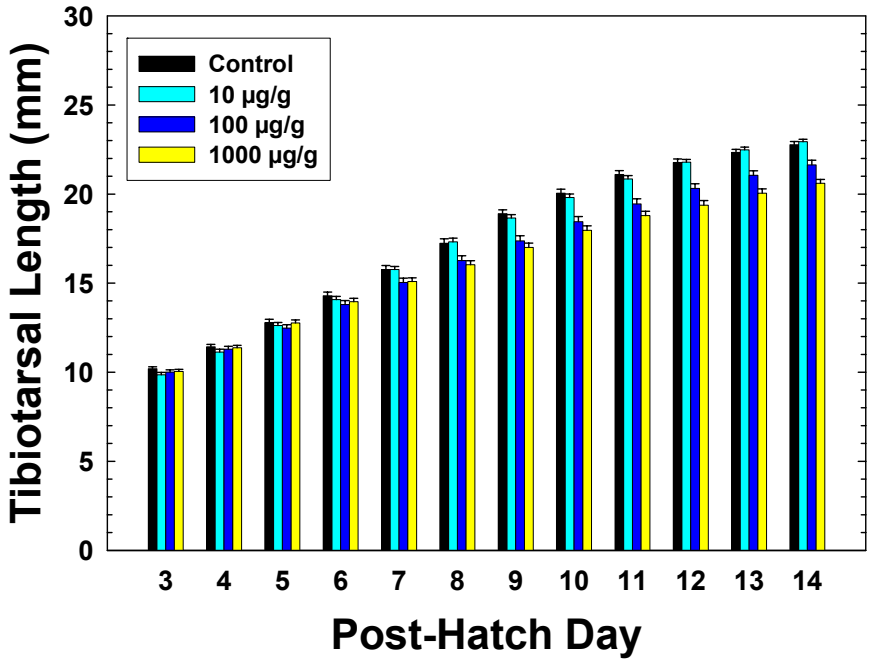


Figure 2. Mean (±SE) tibiotarsal length (mm) of zebra finch chicks
dosed with sodium perchlorate once per day on post-hatch days 3 to 14.

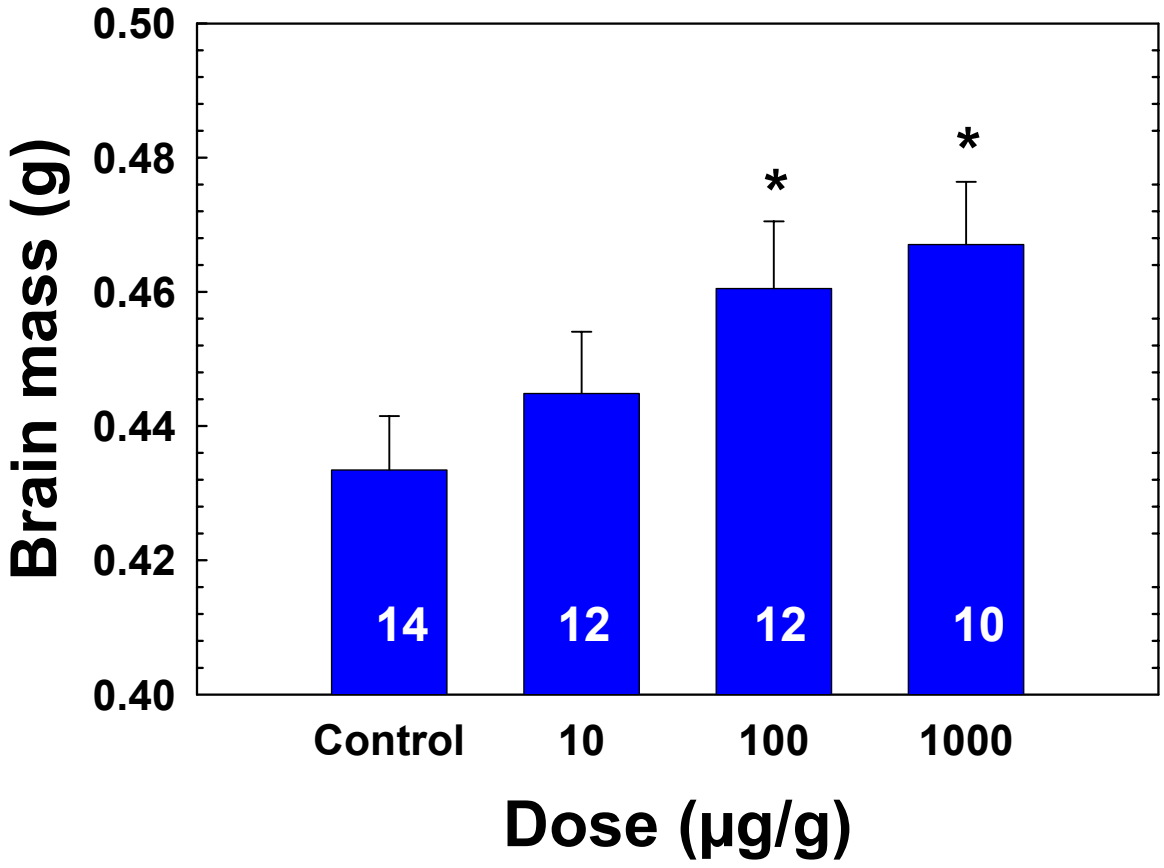


Figure 3. Mean (±SE) brain mass of 72 day-old zebra finches dosed with sodium perchlorate once per day on post-hatch days 3 to 14. Bars with asterisks above them are significantly different than those without asterisks.

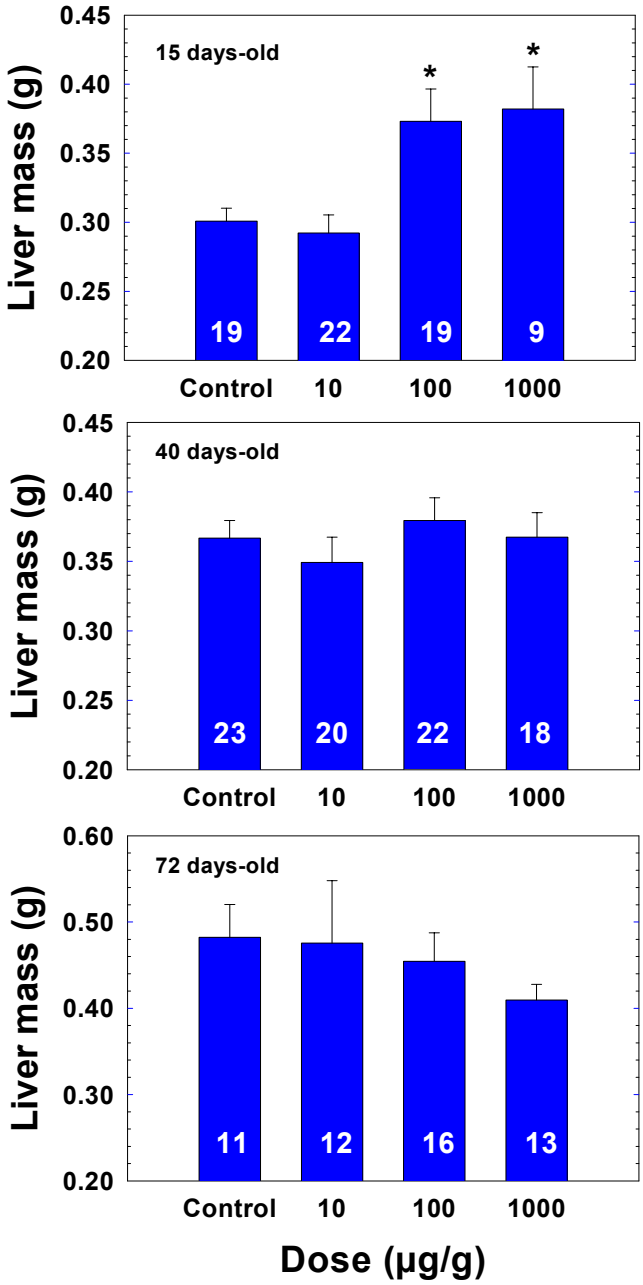


Figure 4. Mean (±SE) liver mass of 15, 40, and 72 day-old zebra finches dosed with sodium perchlorate once per day on post-hatch days 3 to 14. Bars with asterisks above them are significantly different than those without asterisks.

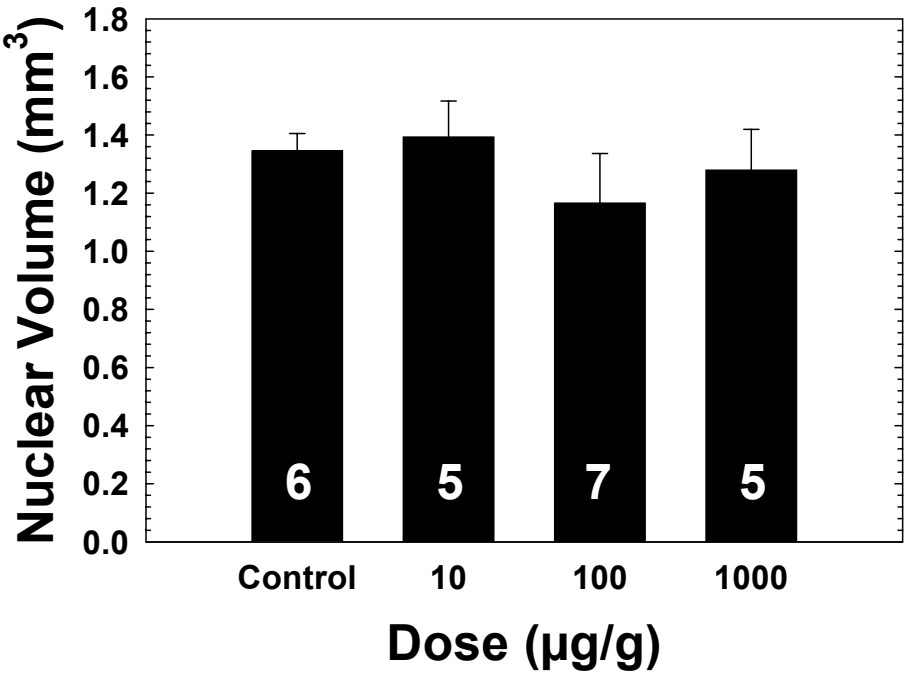


Figure 5. Mean (±SE) volume (mm^3) of the song control nucleus area X in brains of 72 day-old male zebra finches dosed with sodium perchlorate once per day on post-hatch days 3 to 14.

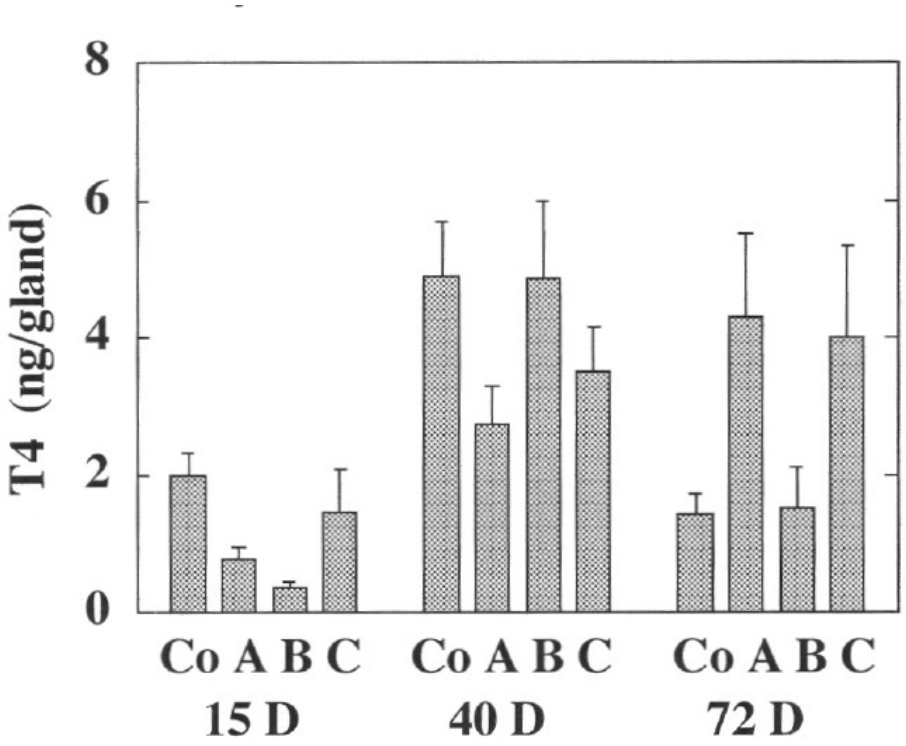


Figure 6. T4 thyroid hormone concentrations in thyroids from zebra finches dosed with perchlorate on post-hatch days 3 to 14. Co, A, B and C represent control (0 ug/g bw) and 10, 100 and 1000 ug/g bw dose groups, respectively. Bars are means and error bars are standard errors.

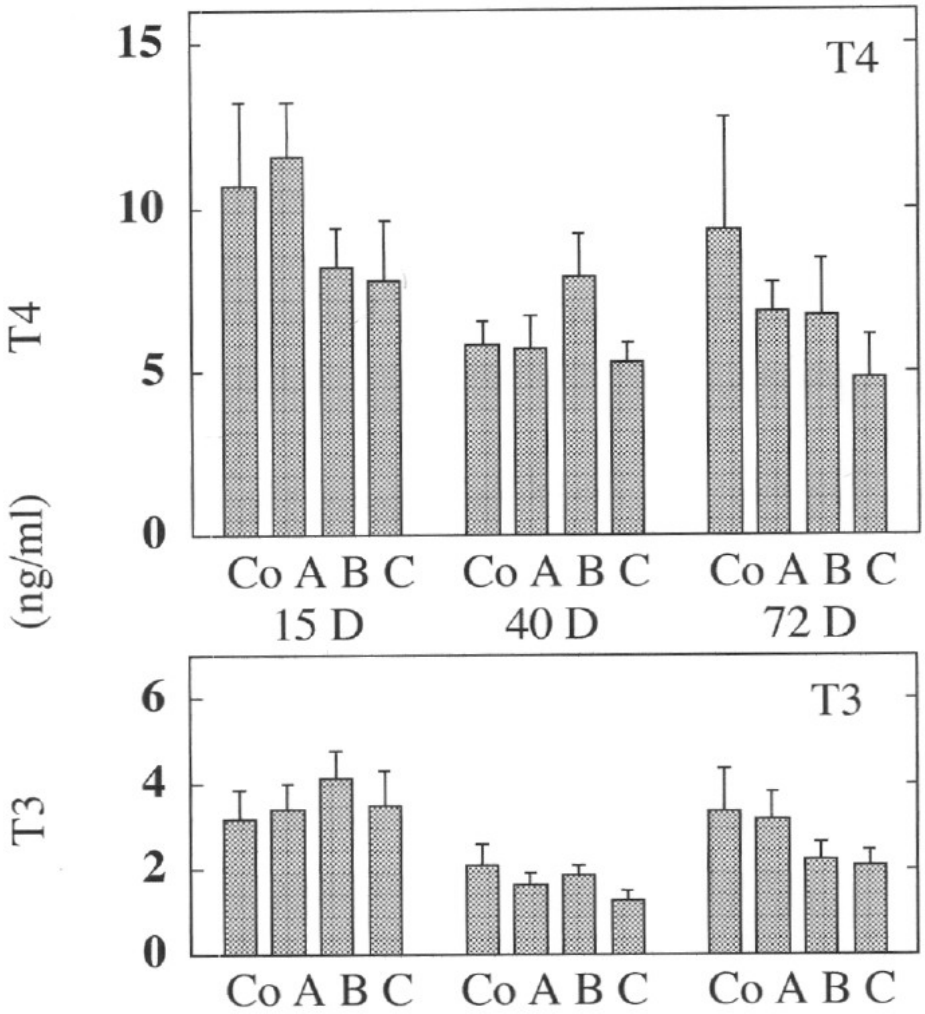
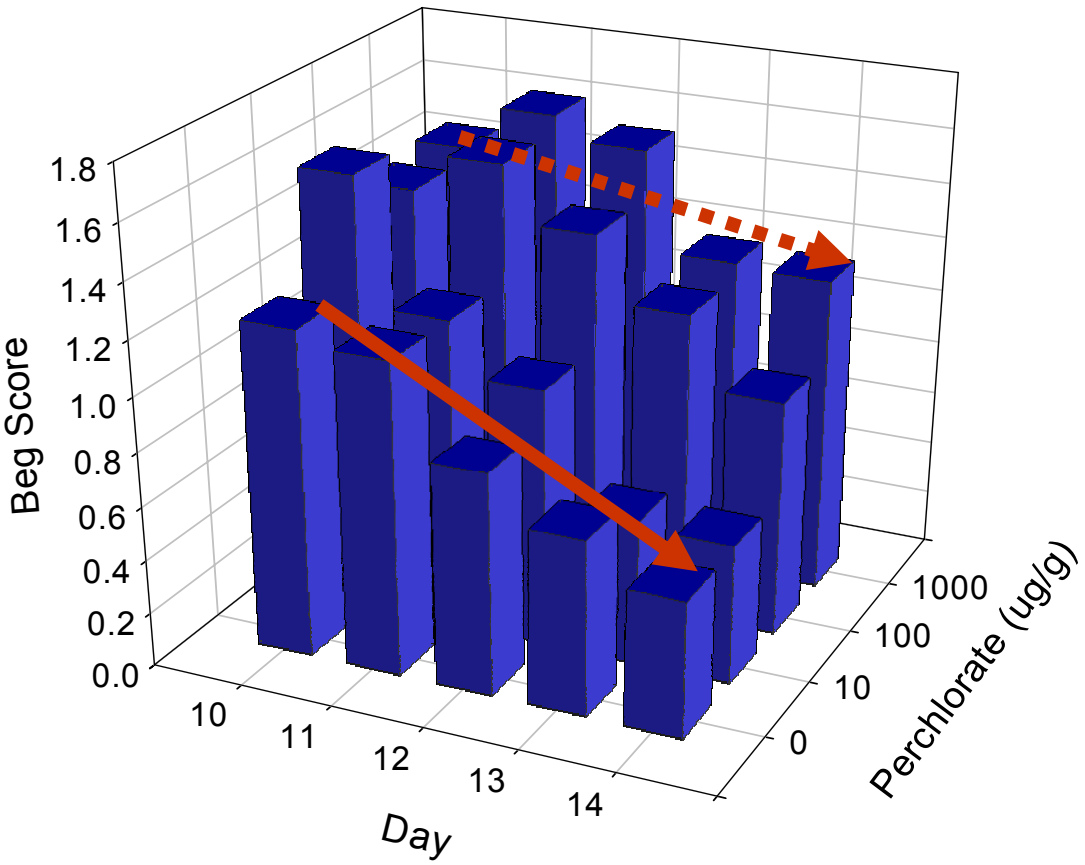
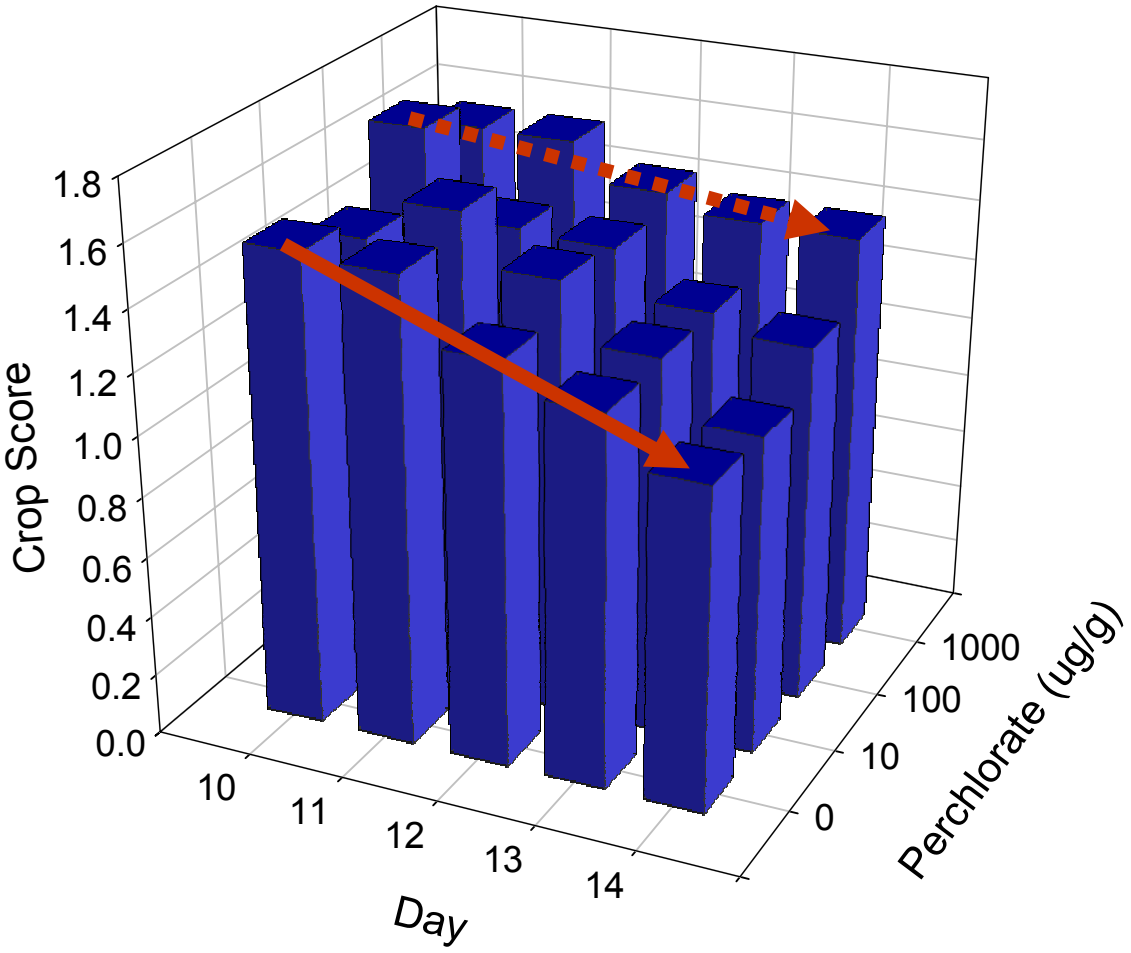


Figure 7. T3 and T4 thyroid hormone concentrations in plasma from zebra finches dosed with perchlorate on post-hatch days 3 to 14. Co, A, B and C represent control (0 ug/g bw) and 10, 100 and 1000 ug/g bw dose groups, respectively. Values are mean + standard error.



Significant differences (P<0.05)	
Age (days)	**Dose groups (ug/g mass)**
11-14	**1000>10, 0**
12, 13	**100>0**
13	**100>10**

Figure 8. Begging behavior in nestling zebra finches (post-hatch ages 10 to 14 days) dosed with perchlorate on post-hatch days 3 to 14.



Significant differences (P<0.05)	
Age (days)	**Dose groups (ug/g mass)**
14	**1000>10, 0**

Figure 9. Crop scores of nestling zebra finches (post-hatch ages 10 to 14 days) dosed with perchlorate on post-hatch days 3 to 14.

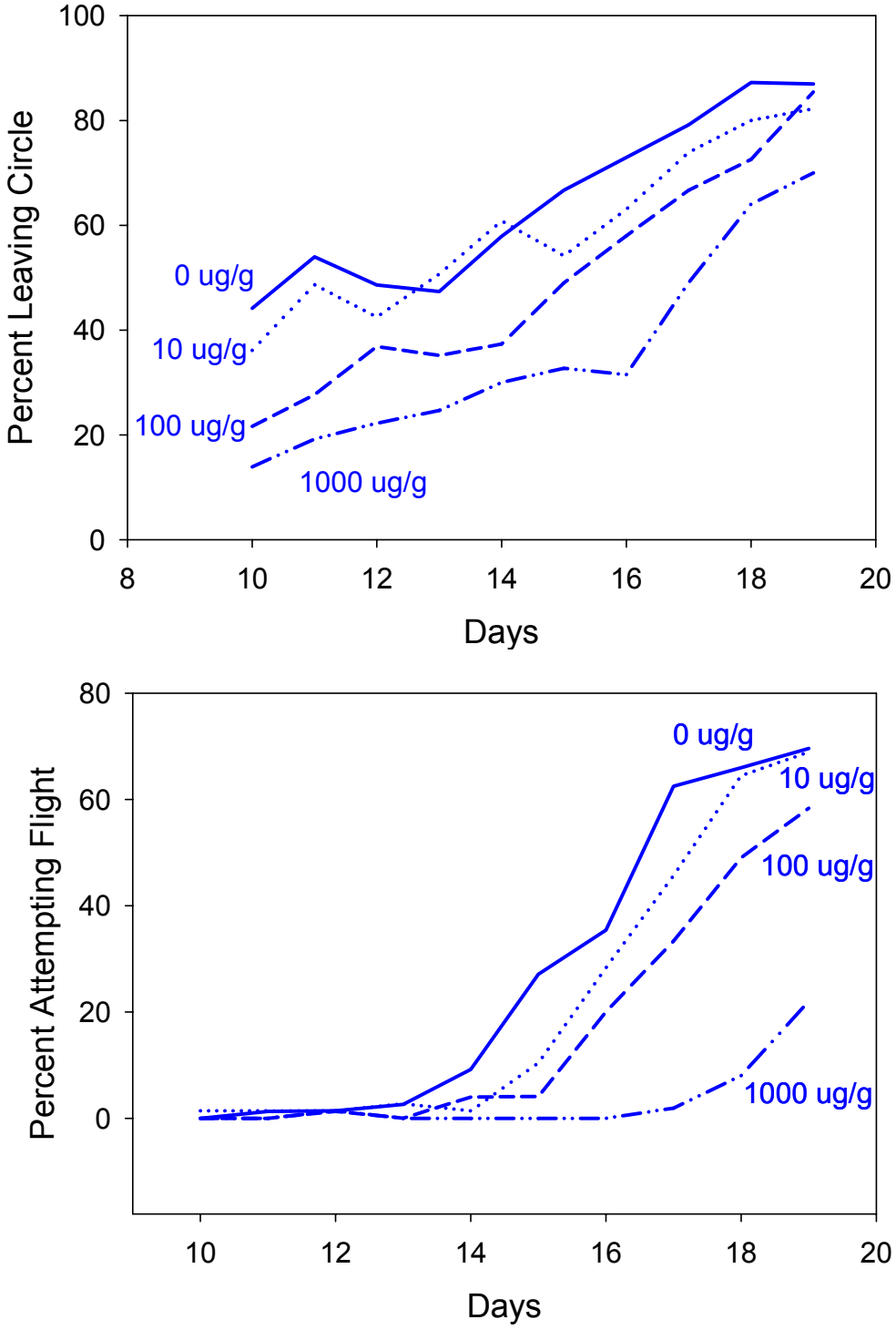


Figure 10. Comparative open field mobility of nestling zebra finches (post-hatch ages 10 to 19 days) dosed with perchlorate on post-hatch days 3 to 14. Upper graph demonstrates percentage of nestlings moving beyond test limits in open field cage. Lower graph documents percent of those nestlings that attempted to fly. All activities occurred within a 30 second period when the birds were introduced into the cage.

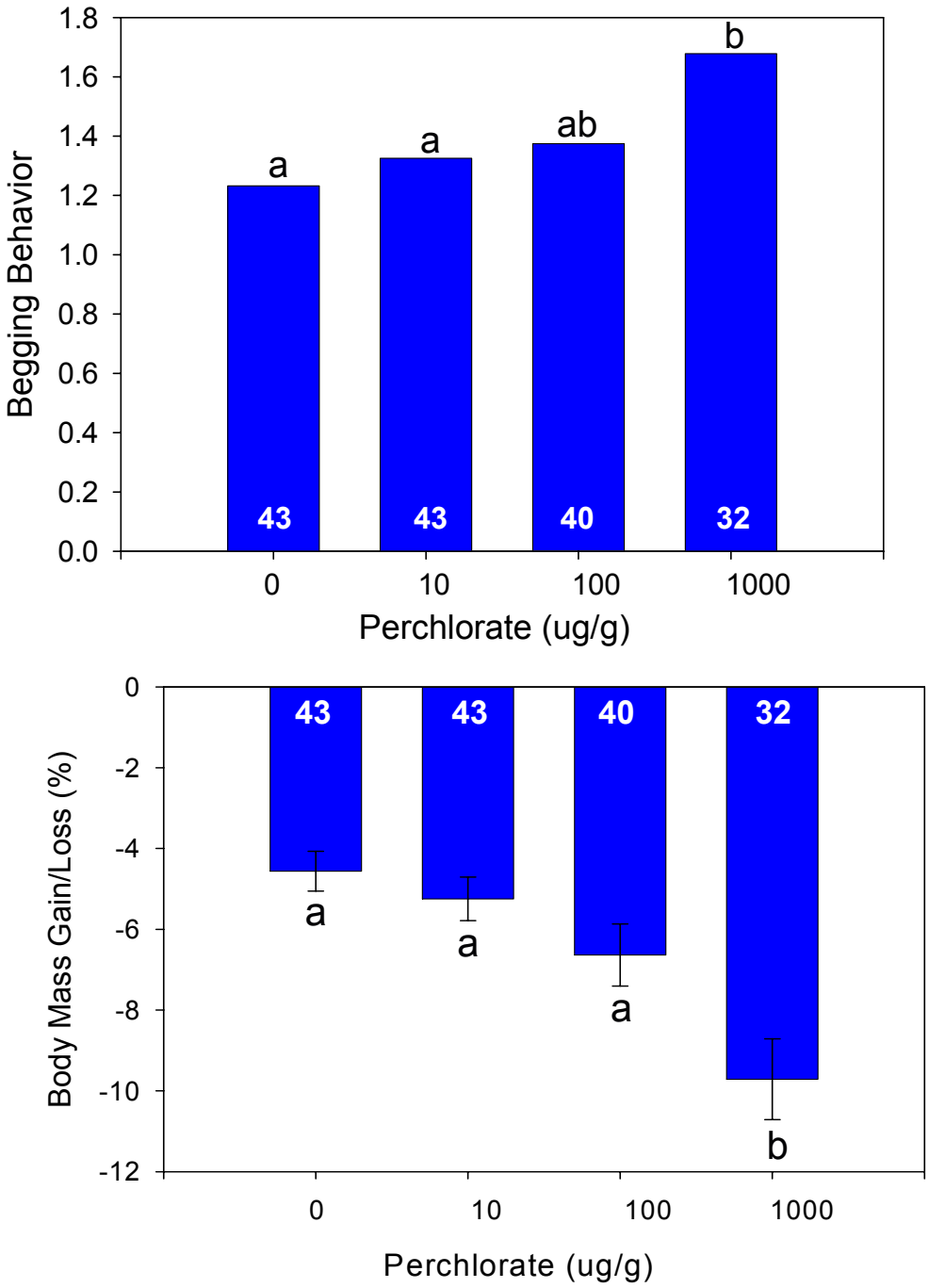


Figure 11. Begging behavior and body mass loss of perchlorate-dosed zebra finch chicks following 8-hr isolation from parents. Post-hatch day 28 chicks were isolated from adults for 8 hours in the presence of food and water. Begging behavior (upper graph) was quantified for first 5 minutes with parents after 8 hr isolation. Body mass change (lower graph) occurred over the 8 hr isolation period.

A FINAL REPORT

ENVIRONMENTAL FATE AND TRANSFER OF PERCHLORATE AND RDX-MESOCOSM STUDY

STUDY NUMBER:	MESO-04-01
SPONSOR:	Strategic Environmental and Research Development Program SERDP Program Office 901 North Stuart Street, Suite 303 Arlington, VA 22203
CONTRACT ADMINISTRATOR:	The Institute of Environmental and Human Health Texas Tech University/TTU Health Sciences Center Box 41163 Lubbock, TX 79409-1163
TESTING FACILITY:	The Institute of Environmental and Human Health Texas Tech University Box 41163 Lubbock, TX 79409-1163
TEST SITE:	The Institute of Environmental and Human Health Texas Tech University Box 41163 Lubbock, TX 79409-1163
RESEARCH INITIATION:	December 2003
RESEARCH COMPLETION:	May 2004

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GOOD LABORATORIES PRACTICES STATEMENT

This study was conducted in accordance with established Quality Assurance Program guidelines and in the spirit of the Good Laboratory Practice Standards whenever possible (40 CFR Part 160, August 17, 1989).

Submitted By:

_____ _____

W. Andrew Jackson Date

QUALITY ASSURANCE STATEMENT

This study was conducted under The Institute of Environmental and Human Health Quality Assurance Program and whenever possible to meet the spirit of the Good Laboratory Practices as outlined in 40 CFR Part 160, August 17, 1989.

Submitted By:

_____ _____

Ryan Bounds Date
Quality Assurance Manager

1.0 DESCRIPTIVE STUDY TITLE: Fate of Perchlorate and RDX in Constructed Wetlands

2.0 STUDY NUMBER: MESO-04-01

3.0 SPONSOR:
Strategic Environmental and Research
Development Program
SERDP Program Office
901 North Stuart Street, Suite 303
Arlington, VA 22203

4.0 TESTING FACILITY: The Institute of Environmental and Human Health
Texas Tech University
Box 41163
Lubbock, TX 79409-1163

TEST SITE: The Institute of Environmental and Human Health
Texas Tech University
Box 41163
Lubbock, TX 79409-1163

5.0 PROPOSED EXPERIMENTAL START & TERMINATION DATES:
Start Date: December 1, 2003
Termination Date: May 30, 2005

22.0 KEY PERSONNEL:
Dr. W. Andrew Jackson, Study Director
Dr. Todd Anderson, Co-investigator
Dr. Kui Tan, Researcher
Darryl Low, Student Researcher

23.0 **STUDY OBJECTIVE/PURPOSE**

- To evaluate the fate of perchlorate and RDX within wetland systems. Wetlands have been proposed as an in-situ passive bioremediation technique for the cleanup of energetics. Natural wetlands frequently serve as interception points for discharging groundwater or surface run-off to waterways. However, little work has been done on the fate and transport of perchlorate and RDX within these systems. Important fate processes include

sorption, plant uptake, microbial transformation, and associated fate of daughter products. Previous studies have shown the ability of bacteria to breakdown RDX and perchlorate as well as aquatic plants ability to uptake RDX (Just and Schnoor, 2004) and perchlorate (Tan et al. 2004).

24.0 TEST MATERIALS:

Test Chemical name: Sodium perchlorate
CAS number: 7790-98-9
Characterization: 99.999% pure
Source: Sigma Aldrich

Test Chemical name: Hexahydro-1,3,5-trinitro-1,3,5-triazine
CAS number: 121-82-4
Characterization:
Source: SRI International

25.0 JUSTIFICATION OF TEST SYSTEM:

Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and perchlorate(ClO_4^-) have been found on numerous military bases at firing ranges, storage facilities, and other sites exposed to explosives and other energetics. As there may be a significant environmental risk posed by these compounds and non-point source cleanups are challenging and economically taxing, installed wetland systems are now being considered. The use of low impact passive technologies such as natural wetland systems to remove RDX and perchlorate from surface water and groundwater may be a promising alternative.

RDX has been a major explosive used by the United States military for over half a century. Widespread and severe contamination has been found at a number of sites across the country. Biodegradation of RDX has been achieved by a number of methods. Anaerobic degradation has been shown to have the greatest degradation potential while aerobic degradation occurs at a much slower rate. The most common degradation pathway is the oxidation of RDX's three nitro groups into nitroso groups then cleaving the ring. Previous studies have observed the uptake of RDX by reed canary grass and RDX degradation within the plant (Just and Schnoor 2004).

Perchlorate has been gaining attention as a contaminant due to its ability to inhibit iodide uptake by the thyroid gland as well as potential health impact at low-dose exposures (US Environmental Protection Agency, 2002). Decreased thyroid hormone production has been seen to be associated with slow heart rate and increased cold sensitivity (Danforth and Burger, 1984).

Currently, there is a scarcity of data on perchlorate and RDX fate in constructed wetlands. RDX uptake by wetland plants has been observed using a small scale

wetlands (Best 1998). However, little work has been done observing both plant uptake and the ability for a wetland system to remediate contaminated surface and groundwater.

26.0 TEST PLANTS:

Species: Graceful cattails (*Typha laxmanil*)

27.0 EXPERIMENTAL DESIGN

Four mesocosms were constructed (dimension of 2 ft L × 1 ft W × 2 ft H) with multiple sampling ports (3, 9, 15, 18 inches below the water sediment-water interface). At the bottom of the microcosm, there was a 3 inch sand layer to distribute and drain the flow. Above the sand, the mesocosms were filled with 15 inches of wetland substrate medium composed of a peat/sand/peat moss mixture (2:2:1 by weight). Physical characteristics of the mesocosms are listed in Table 2.

Graceful cattails (*Typha laxmanil*) were transplanted into the substrate medium. The wetland mesocosms were fed in downflow mode with water containing 25 ppm Cl^-, 5 ppm NO_3^-, and 20 ppm SO_4^{2-} for 2 months in order to establish plant growth. Once established, the exposure to perchlorate and RDX perchlorate began. Two mesocosms were used as duplicates for both RDX and perchlorate separately. Hydrodynamics of the mesocosms were evaluated using conservative tracer studies.

The perchlorate mesocosms were sequentially challenged with influent containing 100 ppb, 1,000 ppb, and 10,000 ppb ClO_4^- and the separate RDX mesocosms were sequentially challenged with 1, 5, or 10 ppm RDX. Each treatment condition lasted at least 3 months until an equilibrium state was reached. Both influent and effluent were sampled weekly as well as at multiple depths within the columns. All samples were analyzed weekly for ClO_4^-or RDX, SO_4^{2-}, Cl^-, and NO_3^-, and tested bi-weekly for total organic carbon (TOC). Plant stems were sacrificed monthly at four locations in the wetland mesocosms to evaluate perchlorate and RDX uptake in plants.

28.0 METHODS:

Water Samples:

Water samples were tested for energetic concentration (ClO_4^- or RDX, TNX, DNX, MNX; and anions and dissolved organic carbon (DOC). Water samples from ClO_4^- challenged mesocosms were filtered with 0.2 µm nylon filters and analyzed for perchlorate and anions using ion chromatography (described below)

and analyzed for TOC using a combustion analyzer. RDX challenged mesocosm samples were filtered using 0.2 μm Teflon filters and the filtered samples analyzed for anions using ion chromatography and for TOC using combustion analyzer, and for RDX were extracted and analyzed using HPLC (described below).

Plant Samples:

ClO_4^- extraction for Plant Samples- Perchlorate plant samples (~1.5g) were cut into small pieces (1 cm^2) and placed in a 99.9°C water bath for 1 hour. Aluminum oxide was added to the extract and after 1 day of incubation at 4C°, the mixture was passed through 0.45 μm membrane filter and diluted 1:5 for analysis.

RDX, MNX, TNX, and DNX extraction for Plant Samples- Plant material was rinsed with DI water to remove surface contamination. Surface moisture on plant tissue can be dried by blotting with paper towels. Plant material was then cut into small pieces and homogenized using a mortal and pestle. Wet plant material (~1g) was transferred to a 15 mL vial and extracted with 10 mL 100 % acetonitrile and thoroughly mixed using a vortex mixer for at least 3 minutes after which samples were sonicated for 1h and centrifuged for 10 min at 3500 rpm.

The sonicated mixture was then passed through florisil cartridges and placed on a 24-port manifold (Supelco, Bellefonate, PA, USA). Before loading samples, florisil cartridges were conditioned with acetonitrile (2 x 5-ml). Filtrates were collected into 10-ml graduated centrifuge tubes. And, the florisil cartridge was rinsed 3 times with small amounts of acetonitrile (3 x 1-ml). Then, the sample was concentrated to 0.5-1.0 mL under nitrogen using a N-EVAPTM111 nitrogen evaporator (Organomation Associates, Inc. Berlin, MA, USA). The final volume was adjusted to 1 mL in the graduated centrifuge tube. The 1 mL extract was finally filtered through a 0.25 μm membrane filter (Millipore, Bedford, MA, USA) and was collected into a GC vial prior to GC analysis.

Analytical:
Water Samples- HPLC was used to analyze water samples. An HPLC (Hewlett-Packard HP 1100) was interfaced with the HP ChemStation software and equipped with a binary pump G1312A, a ultraviolet detector and a autoinjector with a 50 μL loop. The detector was operated at excitation wavelength of 254 nm. Separations were performed with a reverse-phase C18 column (Supelco, Bellefonate, PA).

For energetic compounds RDX, TNT, MNX, TNX and DNX, the mobile phase consisted of 50% acetonitrile and 50% ultra-pure water. The solvent flow rate was 1 mL/min, and the injection volume was 25 μL. Chromatography was performed at room temperature (about 25C). At least three calibration standards were run with each batch of samples to span the expected range of toxicant in samples. Water blanks were also run with each set of samples.

Plant Extracts-A Hewlett Packard 6890 series gas chromatograph (GC) was employed to analyze RDX, MNX, DNX, and TNX. The GC equipped with a HP 6890 autosampler and an electron capture detector was controlled by HP 6890 series ChemStation from Hewlett-Packard (Agilent, Palo Alto, California, USA). Separation was performed on a capillary DB-5 column (30 m x 0.25 mm x 0.25 µm) The GC oven temperature was initially held at 90°C for 3 min, increased to 200° C at a rate of 10° C/min, and then raised to 250° C at 25° C/min, and finally held at 250° C for 5 min. The Injector temperature was kept at 170 °C. The detector temperature was 270°C. The injection volume was 2-µl. The carrier gas was helium (99.9999% pure) at a constant flow-rate of 20 mL/min. The makeup gas for ECD detector was argon methane at a combined flow rate of 60.0 mL/min. The ECD was operated in the constant current mode.

13.0 RESULTS

RDX:

There was significant RDX removal through the wetlands mesocosms for all three loading rates. Removal rates dropped as the loading rate increased with the lowest removal occurring at the 10 ppm RDX loading rate which achieved an 89% decrease in RDX effluent concentration (Figure 1). This was mainly due to microbial degradation, as supported by the disappearance of alternate electron acceptors and lower DOC values with depth. There was a corresponding reduction in nitrate then sulfate with the removal of RDX concomitantly with both electron acceptors. (Figure 2).

RDX was at least partially broken down using the RDX-MNX-DNX-TNX pathway, as seen in previous studies (Figure 3). All of the degraded RDX cannot be accounted for in the byproducts likely due to their instability. Concentration of MNX and DNX were proportional to the loaded RDX concentration. MNX was present in the greatest concentration twenty centimeters below the wetland surface. Past twenty centimeters, MNX concentration was seen to drop corresponding with an increase in DNX with the exception of the highest RDX loading rate (10 ppm) in which DNX did not increase. Interestingly, this was the only loading rate in which TNX was observed at significant concentrations and only at the lowest depths. Total sum of MNX, DNX, and TNX were generally equal to or slightly less than the remaining RDX in the effluent suggesting that exposure may be as significant in cases where RDX laden water may intercept anaerobically active zones.

Plant uptake of RDX also increased with increasing RDX influent concentration (Figure 4). Uptake increased disproportional to increases in RDX influent concentration. Plant RDX concentration increased by three orders of magnitude when the influent water concentration increased by one order of magnitude. This

was not due to bioaccumulation within the plants as there was a rapid increase in plant concentration in the first sampling after increasing the influent. Of the three detected breakdown products only MNX and TNX were found at significant concentrations in the plant material and MNX was typically the dominant species corresponding to the water residue data qualitatively but not quantitatively. This suggests that either the three products are taken up by plants at varying rates or that the plant metabolized the products at different rates. The final plant sampling conducted during the 10ppm RDX exposure separated the plant reed into thirds (bottom, middle, and top). It was found that top third of the reed, while having less plant mass, had much higher RDX concentrations than the lower two thirds. These results suggest that trophic transfer may be an important exposure mechanism for plant consuming organisms.

Perchlorate:

Perchlorate removal was highly successful in the wetlands mesocosm with little to no perchlorate seen in the effluent. Removal rate did drop as the loading rate increased, however the lowest removal was 98.8% for an effluent of 1000ppb ClO_4^- as opposed to the 100% removal (<4ppb) for an effluent concentration of 100ppb ClO_4^- influent concentration (Figure 5). Perchlorate was generally degraded concomitantly with nitrate after which sulfate became the dominant electron acceptor (Figure 6). No inhibition was seen form nitrate even at the highest NO_3^-/ClO_4^- ratios (150).

Perchlorate uptake into the plants was seen to increase with increasing influent concentration. However, unlike the plants exposed to RDX, there was not a disproportional increase in plant concentration. On the contrary, there was only a small increase between plant concentrations when the influent concentration increased from 100ppb to 500ppb ClO_4^- (Figure 7) and concentration factors were in general significantly lower than in previous studies (Tan et al. 2004). This may be due to a deeper rooting depth than for plants previously examined or possibly a higher plant transformation rate than seen for previously studied wetland plants.

17.0 DISCUSSION

Based on our results, constructed wetlands would be a viable method to reduce perchlorate and RDX levels of surface and ground waters. Although the removal rate for both RDX and perchlorate decreased as the loading increased, this could be corrected for by increasing the depth of the wetlands. Wetlands have the potential to remove perchlorate to below action levels and remove RDX to below detection levels. These results also suggest that exposure of ecosystems to RDX in anaerobic environments (e.g. sediments) can produce at least temporary build up of break down products (MNX, DNX, and TNX). In addition plant uptake may pose an increased exposure risk to surface ecosystems beyond that predicted from water analysis alone.

14.0 REFERENCES

Best, Elly P.H. et al. "Environmental Behavior of Explosives in Groundwater from the Milan Army Ammunition Plant in Aquatic and Wetland Plant Treatments. Uptake and Fate of TNT and RDX in Plants." Chemosphere 39 (12) 2057-2072.

Just, Craig L. and Jerald L. Schnoor. "Phytophotolysis of Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) in Leaves of Reed Canary Grass." Environmental Science & Technology. 38 (1) 290-295.

Tan, Kui et al. "Fate of perchlorate-contaminated water in upflow wetlands." Water Research. 38 (2004) 4173-4185.

14.0 FIGURES AND TABLES

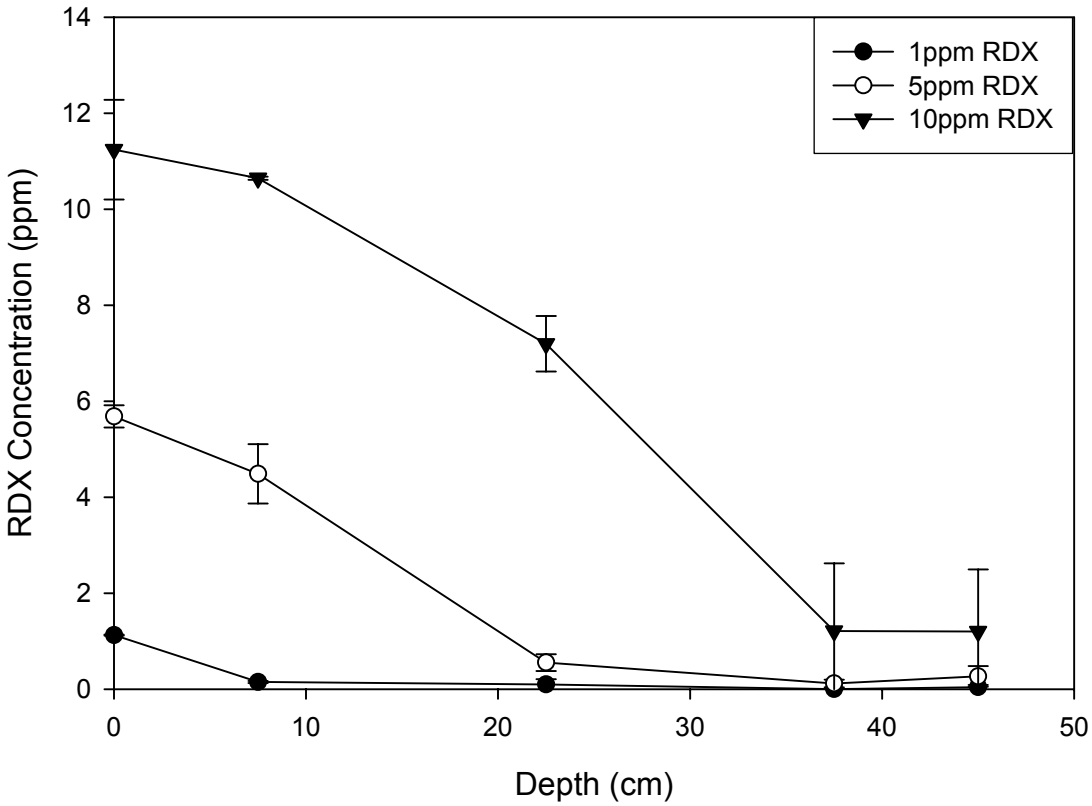

Figure 1: RDX Concentration across wetland depth for various loading rates.

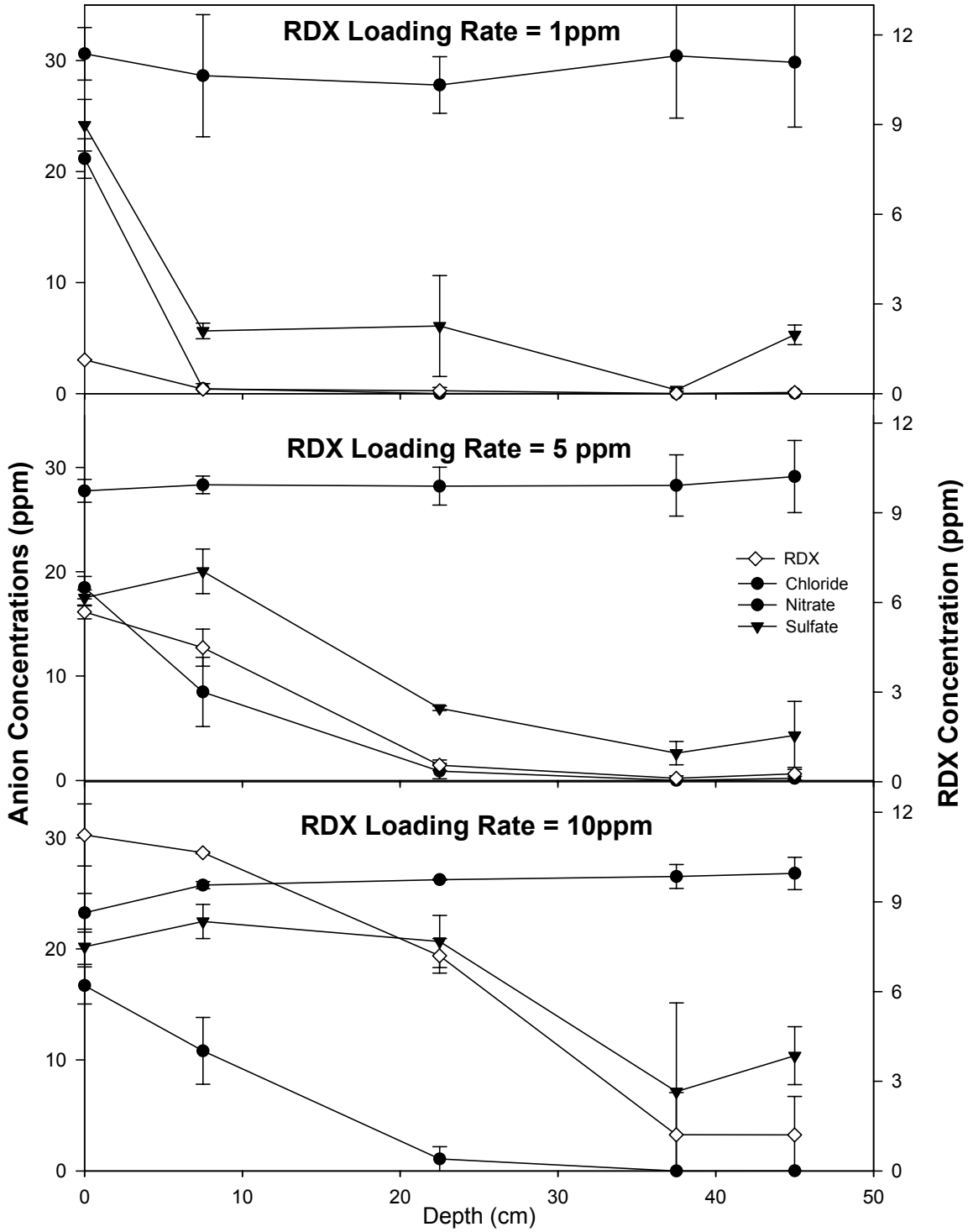

Figure 2: RDX and anion concentrations across wetland depth for various loading rates.

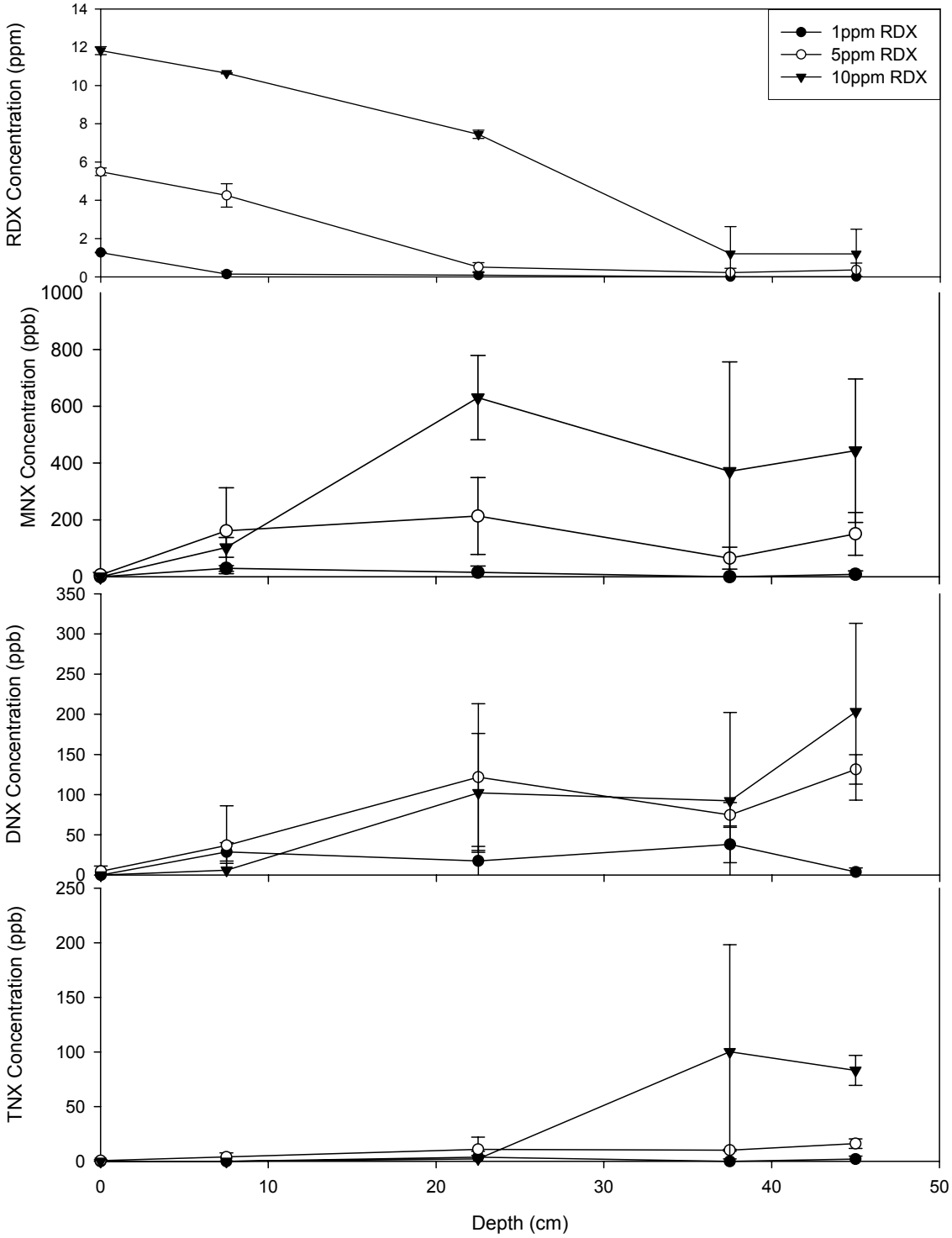

Figure 3: RDX and RDX byproducts MNX, DNX, and TNX concentration across wetland depth for various loading rates.

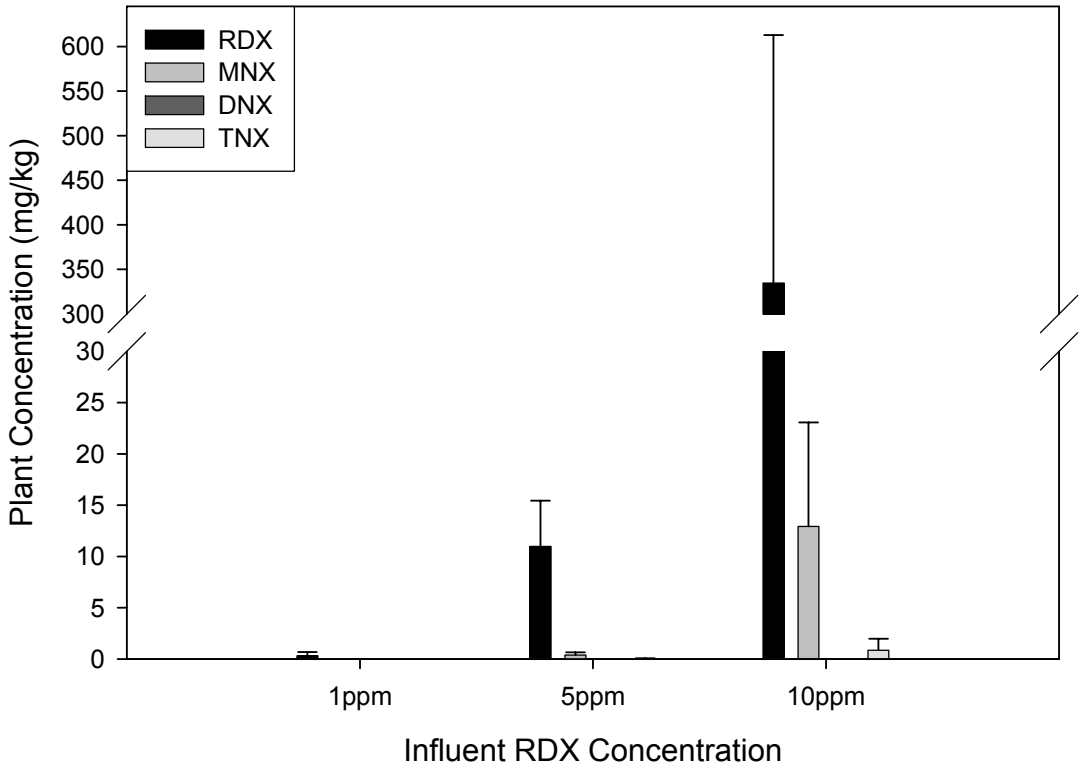

Figure 4: Equilibrium Concentrations of Plant Uptake of RDX and RDX byproducts MNX, DNX, and TNX for various RDX influent loading rates.

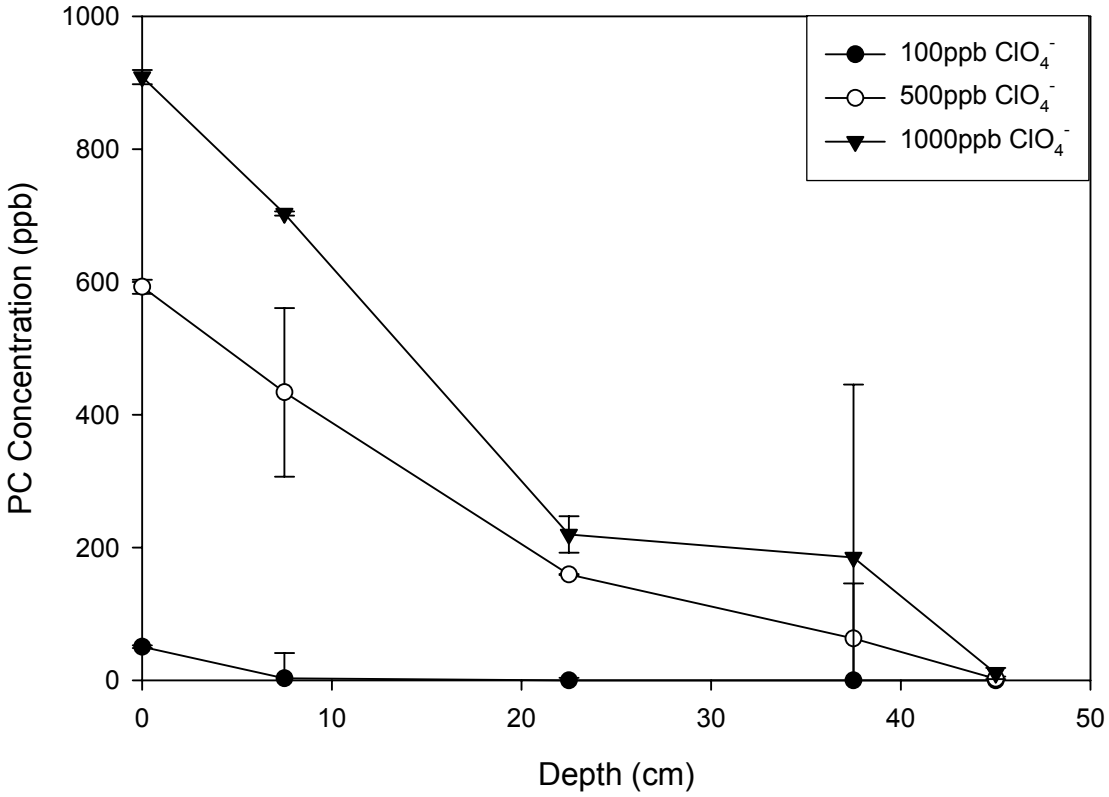

Figure 5: Perchlorate Concentration across wetland depth for various loading rates.

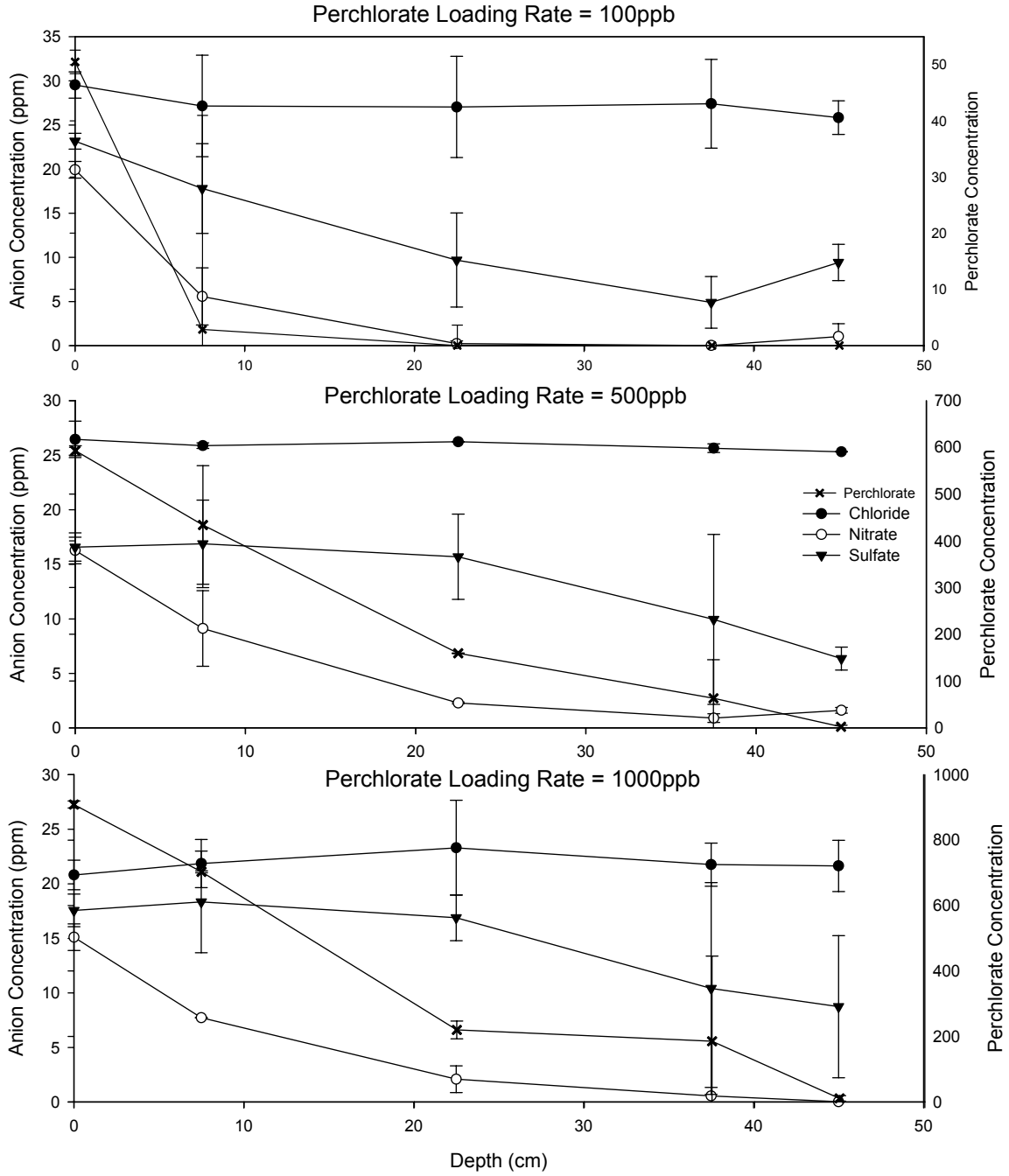

Figure 6: Perchlorate and anion concentration across wetland depth for various loading rates.

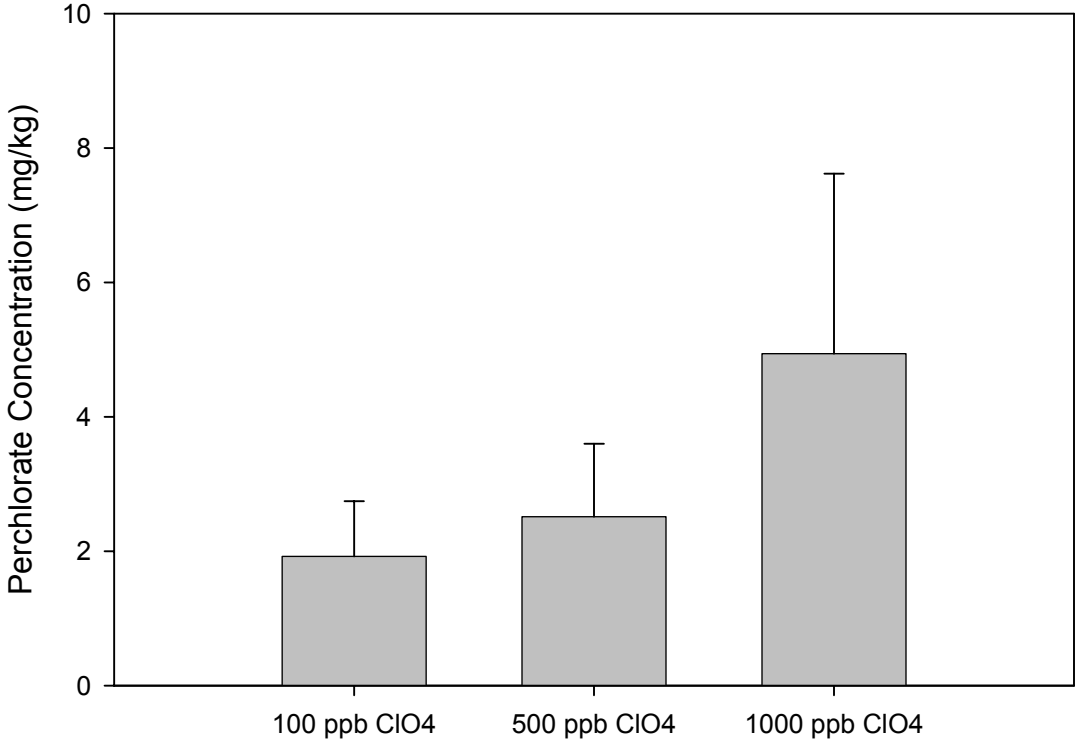

Figure 7: Equilibrium concentration plant uptake of perchlorate for various loading rates.

Table 1: Number of water and plant samples taken for each loading rate

Date	Influent Concentration		Water Samples Taken at Each Depth		Plant Samples Taken	
	RDX (ppm)	PC (ppb)	RDX	PC	RDX	PC
07/05/04-11/01/04	1	100	18	13	4	3
11/8/04-2/21/05	5	500	16	16	4	4
2/28/05-05/10/05	10	1000	11	11	3	3

Table 2. Physical Characteristics of Mesocosms.

Media	**Peat : Sand : Peat moss (2 : 2 : 1 by weight)**
Bulk Density (g/cm3)	**0.71**
Media Porosity	**0.79 ± 0.01**
Hydraulic Conductivity (cm/day)	**70.5 ± 11.9**
Seepage Velocity (cm/s)	**1.18 x 10-4 ± 0.02 x 10-4 cm**
Hydraulic Retention Time (days)	**5**

TITLE: Lethal and Sub-lethal effects of RDX in zebrafish

STUDY NUMBER:	ZEB-04-01
SPONSOR:	Strategic Environmental and Research Development Program SERDP Program Office 901 North Stuart Street, Suite 303 Arlington, VA 22203
CONTRACT ADMINISTRATOR:	The Institute of Environmental and Human Health Texas Tech University/TTU Health Sciences Center Box 41163 Lubbock, TX 79409-1163
TESTING FACILITY:	Texas Cooperative Fish and Wildlife Research Unit Texas Tech University Box 2120 Lubbock, TX 79409-2120
TEST SITE:	Texas Cooperative Fish and Wildlife Research Unit Texas Tech University Box 2120 Lubbock, TX 79409-2120
ANIMAL TEST SITE:	Texas Cooperative Fish and Wildlife Research Unit Texas Tech University Box 2120 Lubbock, TX 79409-2120
RESEARCH INITIATION:	December 2004
RESEARCH COMPLETION:	September 2005

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GOOD LABORATORIES PRACTICES STATEMENT

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Submitted By:

_____ _____

Reynaldo Patiño Date

QUALITY ASSURANCE STATEMENT

This study was conducted under The Institute of Environmental and Human Health Quality Assurance Program and whenever possible to meet the spirit of the Good Laboratory Practices as outlined in 40 CFR Part 160, August 17, 1989.

Submitted By:

_____ _____

Ryan Bounds Date
Quality Assurance Manager

1. Descriptive Study Title:
Lethal and Sub-lethal effects of RDX in zebrafish

2. Study Number:
ZEB-04-01

3. Sponsor:
Strategic Environmental and Research Development Program
SERDP Program Office
901 North Stuart Street, Suite 303
Arlington, VA 22203

4. Testing Facility Name and Address:
Texas Cooperative Fish and Wildlife Research Unit
Texas Tech University
Box 42120, 218 Agricultural Science Building
Lubbock, Texas 79409-2120

5. Proposed Experiment Start and Termination Dates:

Start date:	December 2004
Termination Dates:	September 2005

6. Key Personnel:
Reynaldo Patiño, Testing Facility Management
Sandeep Mukhi, Study Director
Todd Anderson, Assistant Director for Science
George Cobb, Analytical Chemist
Ryan Bounds, Quality Assurance Manager
Ronald Kendall, Principal Investigator

7. Study Objectives/Purpose:
This study examined the acute (lethal) and chronic (sublethal) toxicity of RDX in zebrafish. The goal of this research is to generate a database for ecologically relevant, lethal and sublethal effects of RDX and its metabolites in an important group of aquatic vertebrates, the fishes. Major objectives are to determine (1) the acute toxicity of these compounds in our model organism, the zebrafish; and (2) the sublethal effects of these compounds on somatic and reproductive condition.

8. Study Summary
Hexahydro-1,3,5-trinitro-1,3,5-triazine, a cyclonitramine commonly known as RDX, is used in the production of military munitions. Contamination of soil, sediment, and ground and surface waters with RDX has been reported in different places around the world. Acute and subacute toxicities of RDX have been relatively well documented in terrestrial vertebrates, but among aquatic vertebrates the information available is limited. The first objective of this study was to characterize the acute toxicity of RDX to larval (5-day-old) zebrafish. Mortality (LC50) and incidence of vertebral column deformities

(EC50) were two of the end points measured in this study. The 96-h LC50 was estimated at 22.98 and 25.64 mg L-1 in two different tests. The estimated no-observed-effective-concentration (NOEC) values of RDX on lethality were 13.27 ± 0.05 and 15.32 ± 0.30 mg L-1; and the lowest-observed-effective-concentration (LOEC) values were 16.52 ± 0.05 and 19.09 ± 0.23 mg L-1 in these two tests, respectively. The 96-h EC50 for vertebral deformities on survivors from one of the acute lethality tests was estimated at 20.84 mg L-1, with NOEC and LOEC of 9.75 ± 0.34 and 12.84 ± 0.34 mg L-1, respectively. Behavioral aberrations were also noted in this acute toxicity study, including the occurrence of whirling movement and lethargic behavior. The acute effects of RDX on survival, incidence of deformities, and behavior of larval zebrafish occurred at the high end of the most frequently reported concentrations of RDX in aquatic environments.

The second objective of this study was to assess the chronic (subacute) toxicity of RDX to adult zebrafish. Three-month-old, young adult fish were exposed to measured RDX concentrations of 0, 1 and 9.6 mg L-1 for up to 12 weeks followed by a 15-day recovery in clean water. At 9.6 mg L-1, RDX induced a cumulative mortality rate of about 45 percent over the 12-week exposure, with most mortalities occurring within the first 4 weeks and becoming negligible after 8 weeks. No treatment-related mortalities occurred in the other fish groups. Fish held at 9.6 mg L-1 also showed abnormal feeding behavior during the first 8 weeks of exposure; and somatic measurements indicated that RDX at 9.6 mg L-1 suppressed body weight and condition factor at 4 and 8 weeks of exposure and at 1 ppm, it suppressed body weight at 4 weeks. However, the effects of RDX at 9.6 mg L-1 on feeding behavior were no longer evident after 8 weeks of exposure, and these fish regained normal weight by the end of the 12-week exposure period. Observations of spawning behavior and the presence or absence of larvae in aquaria indicated that RDX at 9.6 mg L-1 inhibited reproductive activity within the first 8 weeks of exposure, but this inhibition was no longer evident after 8 weeks. Histopathological analyses of gonads from fish collected at 12 weeks of exposure did not reveal structural abnormalities. In males, Comet assay of dispersed testicular cells taken at the completion of the exposure period did not indicate the presence of DNA strand breakages related to RDX exposure. RDX and its metabolite, MNX, were detected at 4, 8, and 12 weeks of RDX exposure. The bioconcentration factor for RDX was affected by time of exposure but not by RDX concentration; average combined values were ≤ 1 at 4 and 8 weeks of exposure and > 2 at 12 weeks. RDX and MNX were not detected in fish after a recovery period of 15 days in clean water. The latter observation suggests that RDX and MNX are readily eliminated from the fish. Chronic effects of RDX in aquatic vertebrates need to be further studied for an adequate assessment of the ecological risk of exposure to environmental RDX.

9. Test Materials:

Test Chemical name: Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX)
CAS number: 121-82-4
Characterization: white powder
Purity: 99.9% pure as indicated by supplier
Stability: The chemical was found to be stable at least for 1 week in our test system

Source: Accurate Energetics (McEwen, TN, USA)

Reference Chemical name: Calcium Chloride
CAS number 10035-04-8
Characterization: coarse white powder or mixture with medium size granules.
Purity: certificate of analysis or analytical testing will indicate purity.
Stability: stable
Source: Sigma

Reference Chemical name: Magnesium Sulfate
CAS number: 100-34-99-8
Characterization: colorless crystals
Purity: certificate of analysis or analytical testing will indicate purity.
Stability: stable
Source: Sigma

Reference Chemical name: Potassium Chloride
CAS number: 7447-40-7
Characterization: white crystalline granules
Purity: certificate of analysis or analytical testing will indicate purity.
Stability: stable
Source: Sigma

Reference Chemical name: Sea Salts
CAS number: Not applicable
Characterization: an artificial salt mixture closely resembling the composition of the dissolved salts of ocean water.
Purity: certificate of analysis or analytical testing will indicate purity.
Stability: stable
Source: Aquarium Systems, Inc.

Reference Chemical name: Sodium Bicarbonate
CAS number: 144-55-8
Characterization: white crystalline powder
Purity: certificate of analysis or analytical testing will indicate purity.
Stability: stable
Source: Sigma

Reference Chemical name: Sodium Chloride
CAS number: 7647-14-5
Characterization: white crystalline granules
Purity: certificate of analysis or analytical testing will indicate purity.
Stability: stable
Source: Sigma

Reference Chemical name: Ultrapure water with added salts needed by fish will be used as reference solution to which negative reference material or test material will be added for treatments.

CAS Number: Not applicable
Characterization: water quality will be tested by chemical analysis and pH will be monitored regularly.
Purity: ultrapure
Stability: stable
Source: City tap water or steam plant deionized water that has been run through a carbon filter and a de-ionizer to convert it to ultrapure water. Sea salts (60-240 mg/L) will be added to this water, or a modified FETAX will be made.

10. Justification of Test System:

The cyclic nitramine, hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), is widely used as an explosive in commercial and military operations. It is believed that the large-scale manufacture, use, and improper disposal of RDX have led to severe contamination of soil and ground water by this compound and its metabolites (Haas et al. 1990; Sunahara et al. 1999). RDX in natural soil environments is metabolized to different other compounds (Sheremata et al. 2001). RDX metabolites may include hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX), hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX), and hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX). RDX and its metabolites do not strongly adhere to soil particles, and there is a high potential for leaching from contaminated soils into surface waters. Among fishes, the number of species for which the acute (lethal) toxicity of RDX has been tested is small (Bentley et al. 1977; Hovatter et al. 1997). In addition, no information is currently available for fishes on the sublethal effects of RDX and its metabolites. Thus, studies of the acute and subacute effects of these compounds in fishes are clearly needed to assess their impact on the aquatic biota.

The model organism for the present study is the zebrafish (*Danio rerio*). Zebrafish are frequently used in biomedical research and have the advantage of having available a wealth of information concerning its genetics and developmental biology. Also, the zebrafish genome sequencing project and the availability commercial cDNA microarrays allow for the convenient application to this species of current tools in molecular biology. Further, zebrafish are easy and economical to maintain in the laboratory, and we have prior expertise and experience using this species for toxicological research (Patiño et al., 2003).

11. Test Animals:

Species:	*Danio rerio*, Zebrafish
Strain:	Wildtype
Age:	Five-day-old larvae produced in the laboratory and sub-adults (2 months of age at the time of purchase).
Number:	2415, mixed sex population.
Source:	Two and one-half month-old fish were purchased from Aquatic Research Organisms (Hampton, NH, USA).

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12. Procedure for Identifying the Test System:

For acute toxicity study, 15 5-day old juveniles were exposed to RDX for 96 hours in 250-mL beakers following a static-renewal procedure (ASTM). At minimum, each beaker were labeled with the AUP number, the project number, concentration, chemical, replicate #, and date initiated. Fish were not fed during the exposures, and

water quality was maintained by replacing at least fifty percent of the water volume daily with water containing the appropriate concentration of RDX.

For the subacute toxicity study, 2.5-month-old zebrafish were housed in 10-gallon aquaria (filled with 28 liters of zebrafish water). Each tank was labeled with the AUP number, the project number, concentration, chemical, replicate number and date initiated. Following an acclimation period of approximately 2 weeks, RDX was added to aquaria at the appropriate concentrations and the exposure was continued for a total of 12 weeks. Fish were fed with frozen or live brine shrimp or other prepared diet twice daily. Feeding behavior and animal health (external appearance) was monitored daily. Water temperature, pH, salinity, and dissolved oxygen were recorded daily. One half of the water volume (14 L) was replaced once weekly and total ammonia-N level was monitored once weekly. At each sampling time, fish were euthanized in a lethal concentration of anesthetic (1.0 g/L MS-222) following TCFWRU SOP AF-3-03. Samples thus collected were fixed in Bouin's fixative until histological processing, or snap-frozen in liquid nitrogen and stored at -80°C until measurement of whole-body RDX concentration. RDX levels in test solution and in sample tissue were measured by HPLC and GC respectively.

13. Experimental Design Including Bias Control:

The acute toxicity study was designed according ASTM protocol (ASTM , 1997). For the subacute toxicity study, two environmentally relevance concentrations were chosen: 1 and 10 mg L-1 nominal concentrations. Control tanks (containing normal water) were also included in this experimental design. Young adult zebrafish were approximately 3 months of age at the time the exposures were initiated. A static-renewal procedure was followed for the subacute study. (For detail procedures for both acute and subacute studies, see descriptions provided in subsequent sections of this report).

The total number of fish required for the acute and subacute toxicity studies was calculated as follows:

1. Acute Toxicity Test: (LC50 at 96 hours)

Range-finding tests: 10 larva/replicate x 1 replicate per treatment x 8 treatments x 1 chemical =80
Definitive tests: 10 larva/replicate x 5 replicate per treatment x 8 treatments x 1 chemical x 2 test =800

An additional 5 larva were added to each replicate to compensate for losses due to normal mortality during larval development. Therefore, the extra number of fry required for the above two tests was 4+400 = 440

The total number of fry required for acute toxicity test = 80+800+440 = 1320

2. Sub-acute Toxicity Test:

45 juveniles*/replicate x 3 replicates x 3 treatments x 1 chemical = 405

An additional 10 fish was added to each replicate to compensate for losses due to normal mortality during development. Therefore, the extra number of juveniles required for above test = 90

Total juveniles required for sub-acute tests= 405+90 = 495

The total number of animals needed for the acute and subacute tests is: 1320+495 = 1815

*45 Juveniles = [(10 fish for histology + 5 fish for RDX in body)/sampling time] X 3 sampling times. (Sampling time and method are described in subsequent sections)

14. Methods

14.1 Acute Toxicity of RDX

14.1.1. Chemical Analysis

Water samples were analyzed for actual RDX concentrations by HPLC. To construct the standard curve, standards (20 - 10,000 µg L-1) were prepared from the standard stock solution (1000 mg L-1) in 50:50 acetonitrile:water (zebrafish-water). At least 9 standards were prepared for calibration. Each analysis sequence began with injections of at least three continuing calibration standards within the calibration curve range. Single continuing calibration standards were run with every 10-15 samples in the sequence, and responses were averaged into the existing standard curve. If the instrument response of the continuing calibration standards had changed by 15 percent, then a new standard curve was developed using all calibration standards. Before injection, water samples were diluted with 50 percent acetonitrile and filtered through a 0.2 µm PTFE syringe filter (Fisher Scientific, NH, USA) into the autosampler vials.

A Hewlett-Packard 1100 HPLC instrument was used with a UV detector set at a wavelength of 254 nm. Separation were performed with a Supelco C18 column (4.6 x 25 mm, 5-µm packing, Supelco, Bellefonate, PA, USA) using a mobile phase of 50 percent acetonitrile to 50 percent water. The solvent flow rate was 1 ml min-1, and the injection volume was 15 µl. Each run took 7 min. Peak areas were obtained using a HP 3390A integrator.

14.1.2. Fish breeding and larval rearing

Protocols for the use of animals in this study were reviewed and approved by the Texas Tech University Animal Care and Use Committee (Lubbock, TX, USA). Larvae for the acute toxicity study were obtained from a colony of adult zebrafish maintained on campus. Colony fish were purchased from Aquatic Research Organisms (Hampton, New Hampshire, USA) and reared in 200-L aquaria with external biofilters. Water quality parameters were maintained at recommended levels for zebrafish (pH 6.5 - 8.0; 28 ± 2°C; 14:10 light:dark cycle; unionized NH_3 <0.01 ppm). Male and female broodstock zebrafish were maintained separately and fed adult brine shrimp twice daily to satiation. Male and female fish were placed together for breeding the evening before embryo collection according to methods described by Patiño et al. (2003). Fertilized eggs

(embryos) were collected 3 h after lights-on the following morning. Embryos were washed several times with zebrafish-water to remove fecal matter and other debris, and were then counted into a 1-L beaker (200 eggs per beaker) containing 400 ml of zebrafish-water (temperature 27-28°C). Beakers were maintained at 27-28°C in a water bath, and other parameters were kept optimal for zebrafish as described above, except that the light:dark cycle was 12:12 h. Unfertilized (opaque) eggs were removed from the beakers 12 h after spawning. Fifty percent of the water was exchanged with fresh zebrafish-water (preheated to 27-28°C) everyday after removing any dead eggs or egg shells from the hatched fish. Most eggs hatched 3-4 days after fertilization. Unhatched eggs were removed and discarded on day 4 after fertilization to maintain homogeneity in the age-since-hatching among larvae. Five-day-old larvae were used for acute toxicity tests.

14.1.3. Range-finding test

The 96-h acute toxicity of RDX was determined according to guidelines provided by the American Society for Testing and Materials (ASTM 1997). Five-day-old larvae were assigned to seven RDX treatments and one control. The nominal concentration of RDX in the highest treatment was designed to be 40,000 µg L-1 and the lowest treatment group was 0.04 µg L-1. There was a 10fold-difference between each treatment concentration except the 0.04 µg L-1-group. The RDX concentration in treatment beakers was verified by HPLC measurement. As the detection limit of RDX in test solution was 20 µg L-1, RDX could be detected only in the highest four treatment concentrations. The mortality data generated from this preliminary range-finding test was utilized in designing the definitive tests.

14.1.4. Definitive Test 1

A randomized complete block design was implemented with 40 experimental units (250-mL exposure beakers), eight treatment levels, and five replicates per treatment. Within each of the water baths, each beaker was randomly assigned one of the eight treatments. Each beaker contained 100 mL of zebrafish water and 15 larvae. The larvae were acclimatized to the beaker for 24 h before starting the exposure. To start the exposure, approximately 95 percent of water was removed from each beaker and replaced with treatment water containing the appropriate RDX concentrations. In subsequent days, approximately 95 percent of water volume was replaced daily with freshly prepared treatment solutions. Water quality parameters (pH, temperature, unionized ammonia, and dissolved oxygen) were measured daily. The temperature of the water bath was used as indicator of the temperature of the water in the beakers. Dissolved oxygen was measured with a YSI 85™ meter (YSI Inc., Yellow Springs, OH, USA) and pH was measured with a pH Tester 2™ (Oakton Instruments, IL, USA) before the daily water exchanges. Water samples were collected daily from each beaker before the water exchange for determination of actual RDX concentrations and unionized ammonia content. Dead larvae were counted and removed daily before exchanging the water. At the end of the 96-h exposure period, the total number of live fish was determined in each beaker. The fish were then euthanized with 1 g L-1 of MS-222 (Sigma, St. Louis, MO, USA) and waste water was disposed in an appropriate waste container.

14.1.5. Definitive Test 2

The design for this test was similar to that of the preceding *Definitive Test 1*. Results from the preceding tests indicated that the variability among the measured RDX concentration for each replicate within a treatment was narrow and close to the measured RDX concentration in the working solutions. Thus, RDX concentrations for this test were not measured in each beaker but instead were measured only in the working solutions that were used as source for beaker water. All fish handling and monitoring procedures for this test were as described earlier.

From the preliminary observations of the *Definitive Test 1*, it was determined that RDX caused vertebral column deformities in the developing larvae. These observations prompted us to determine the incidence of vertebral column deformities in *Definitive Test 2*. Mortality rates in second test were also recorded and used for calculation of LC 50 for this definitive test. Data collected on vertebral deformities were used to determine the EC50. Behavioral effects were also noted as gross observations. Larvae were observed for gross behavioral abnormalities at 12 hour intervals, and at the end of the 96-h exposure all surviving fish were euthanized in MS-222 (Sigma, St. Louis, MO, USA) and preserved in 10% neutral buffered formalin (Sigma, St. Louis, MO, USA). The overall incidences of vertebral column deformities (Brown and Nunez 1998; Silverstone and Hammell 2002) were determined on the survivors. For this purpose, all larvae per beaker were observed under a stereo microscope to determine the percentage of those showing vertebral deformities.

14.1.6. Data Analyses

Data were subjected to probit analysis to calculate LC50 and EC50 with 95 percent confidence limits (CL) using SPSS (SPSS Inc., Chicago, IL, USA) after logarithmic transformation of RDX concentrations. For the *Definitive Test 1*, the RDX concentrations measured for each individual beaker over the 96 h period (24 h interval) were averaged to obtain the mean for each beaker, and the average of the five replicate beakers was used to represent the concentration of each treatment. For the *Definitive Test 2*, the concentrations of the working solutions prepared daily for each treatment group were averaged over the 96-h to obtain the respective treatment concentrations. Percent values for mortality and deformities were transformed by arcsine of square-root before application of one-way analysis of variance (ANOVA, (α= 0.05); and Duncan's multiple range test was used (α= 0.05) to determine the Lowest-Observed-Effect Concentration (LOEC) and the No-Observed-Effect-Concentration (NOEC) for lethality and vertebral deformities. These statistical analyses were conducted using the Statistica® software package (StatSoft, Tulsa, OK, USA). A linear regression analysis was done to assess correlation between the percent vertebral deformities and the water borne RDX concentration. Behavioral observations were qualitatively assessed.

14.2. Subacute toxicity study

14.2.1. Experimental animal

Protocols for the use of animals were approved by the Texas Tech University Animal Care and Use Committee (Lubbock TX, USA). Two-and-one-half-month-old, wildtype zebrafish (*Danio rerio*) were obtained from a local vendor and acclimatized in our laboratory for about 2 weeks prior to experimentation. Animal husbandry procedures for this study were as described by Mukhi et al. (2005). Briefly, each treatment unit

(tank) consisted of a 10-gallon aquarium filled with 30 L of zebrafish-water and fitted with two hand-made, glass-based internal biofilters. A water current through the filter was maintained by airflow via glass pipette. Water quality parameters were maintained at recommended levels for zebrafish (pH 6.0-8.0, 26-28.5 ºC, 12/12 light/dark cycle). Fish were fed until satiation either with adult frozen *Artemia* or Tetramin® flakes (Tetra Sales, Blacksburg, VA, USA) twice daily. On every evening, leftover food and fecal material were removed by siphoning. Water quality (pH, temperature, dissolved oxygen, specific conductivity, and salinity) was measured daily and ammonia-N was measured at least twice weekly.

14.2.2. Experimental design and treatment

In addition to a control group, two environmentally relevant concentrations (nominal) of RDX were chosen for this study: 0 (control), 1 and 10 mg L-1. Each treatment was conducted in triplicate. Zebrafish were randomly distributed to each of 9 experimental tanks. A static-renewal exposure method was followed. Every week, either 50 percent of treatment water twice, or 100 percent of treatment water once, was renewed with fresh treatment water. Treatment work solutions were prepared in 9 separate tanks (10-gallon aquaria) situated on a rack above the corresponding treatment tanks. The required amount of test material (RDX slurry) was poured into each of the overhead tanks at least 24 hours before water exchange. These tanks were aerated (to create water circulation and help dissolve RDX) and heated to the appropriate temperature before adding to the treatment tanks. On the day of water renewal, either 50 or 100 percent of the treatment water was removed by siphoning and disposed appropriately, and the appropriate volume of the fresh exposure water was added to the treatment tank by gravity flow. The volume of water exchange was decided depending upon measurements of water quality such as unionized ammonia, turbidity or algae infestation. For verification of actual RDX exposure concentration, water samples (2-3 ml) were collected from the treatment tanks each time before and after each water exchange. Fish feeding and swimming behaviors were observed daily and any sign of abnormal behavior was recorded. Fish breeding behavior (males chasing around females at surface of the biofilters) was closely monitored, and the presence of any embryo or larvae in or around the biofilters was also recorded. Mortality of fish, if any, was recorded and dead fish were removed and disposed appropriately. Sampling was done at 4, 8 and 12 weeks of exposure. At each sampling time, 10 fish from each aquarium were collected and rinsed twice in zebrafish water, euthanized with MS-222 solution (1 mg L-1), and 5 fish were either fixed in Bouin's fixative (for histology) or snap-frozen in liquid nitrogen (for RDX measurement). Fish weight and fork-length were recorded at the time of sampling. Condition factor for each fish was calculated according to the formula 100,000 x body weight (g)/length^3 (mm^3).

14.2.3. RDX elimination

At the completion of the 12-week exposure period, 5 fish per tank were rinsed in zebrafish water several times and transferred to new respective tanks. Fish were held in these tanks for a total of 2 weeks. All standard husbandry practices for zebrafish were applied in this study. Water samples were collected during the 2-week period to check for the presence of RDX in water. One water exchange was conducted at the end of the

first week. After 2 weeks, fish were euthanized and snap-frozen in liquid nitrogen for verification of RDX and its metabolites in whole-body homogenates.

14. 2.4. Chemical analysis in treatment water and stock solution

Water samples were analyzed for actual RDX concentrations by HPLC according to Mukhi et al. (2005). Our original study design did not include measurement of RDX metabolites in the treatment solution. However, since MNX was detected in the tissue extract (see Results section), we decided to measure possible contamination of our RDX stock with RDX metabolites. For this purpose, liquid chromatography and mass spectrometry (LC-MS) were used.

14.2.5. RDX and MNX extraction and analysis

A preliminary analysis showed that only RDX and MNX could be detected in tissue extracts from RDX-exposed fish. Other RDX metabolites such as TNX and DNX were not observed. Therefore, the tissue extraction procedure and analysis methodology described here is for RDX and MNX only. The procedure for extraction, cleanup and analysis of RDX and its metabolites in fish tissue was according to Pan et al. (2005) with some modifications. Briefly, 5 fish per treatment replicate were pooled and dehydrated by grinding with 8-10 g of Na_2SO_4 (which also served as dispersing agent). To estimate the percent recovery for RDX and MNX, extracts from untreated fish were spiked with standard solutions. Ground tissue samples were loaded into 22-ml cells and extracted with 20 ml of acetonitrile using a Dionex ASE 200 extractor (Salt Lake, UT, USA). Extracts were evaporated to 1-2 ml using a rotary evaporator and subsequently cleaned using Florisil® cartridge (Supelco, Bellefonate, PA). Extracts were evaporated under nitrogen gas and concentrated to a final volume of 1 ml. The samples were analyzed by gas chromatography with an electron-captured-detector (HP 6890 series GC-ECD Agilent, Palo Alto, CA, USA). Each analysis sequence began with injections of at least three calibration standards that spanned the needed calibration range. The limit of detection of the instrument was 17 and 20.6 ng/g for RDX and MNX respectively. The presence of RDX and MNX in tissue extracts was confirmed by LC-MS.

14.2.6. Gonad histology

Whole fish were placed in the Bouin's fixative for 48 h after euthanization and processed for histological observations using standard techniques. Seven-micron sections of the anterior region of the gonad were cut and stained with hematoxylin and eosin and observed under a microscope. In females, the presence or absence of different stages of oogenesis was recorded for each fish. Specific stages recorded were pre-vitellogenic (perinucleolar), vitellogenic, post-ovulatory and atretic follicles. In males, the presence or absence of spermatogonia, spermatocytes, spermatids and sperm was recorded. Also, the percent area of testicular cross-sections occupied by sperm was determined using digital images taken with an Olympus digital camera (DP70) attached to a compound microscope and Image-Pro® Express software (Media Cybernetics, Silver Spring, MD, USA). Gonadal preparations were inspected for the presence of gross histopathological features (Blazer, 2002).

14.2.7. Comet assay

A comet assay was used to assess the genotoxic effect of RDX in zebrafish. Although the liver was the original target tissue for this assessment, the small size of this organ in the experimental fish made its dissection difficult. The testis was used as replacement tissue. Testicular samples were collected at 12 weeks of RDX exposure. A testicular cell suspension was prepared from freshly euthanized zebrafish and Comet assay (single cell gel electrophoresis) was performed using a commercial kit according to the manufacturer's protocol (CometAssay™, Trevigen). Slides were stained with CYBR® green and observed under fluorescence microscope at maximum excitation and emission of 494 nm and 521 nm, and at 200X magnification. For each sample analyzed, 80-100 cells were randomly chosen and counted as comet positive or negative. One male fish per replicate aquarium was analyzed, for a total of 3 fish per treatment. The assay was validated using UV-irradiated hepatocyte cell line (HepG2) as positive control.

14.2.8. Data analysis

As there was no substantial difference in the RDX concentration (mg L-1) before and after water exchange in each tank, the average of these concentrations were calculated from all measurements taken for each tank per week. For all somatic and reproductive condition parameters, individual fish values within each aquarium were averaged to obtain tank values. Comet assays were conducted for one male fish per aquarium, and RDX bioaccumulation was measured in pools of five fish per aquarium. Thus, in all cases, sample size per treatment for statistical analyses is the number of tank replicates (n = 3).

Unless otherwise noted, the effects of waterborne RDX (concentration and exposure time) on somatic condition and reproductive condition and on RDX bioaccumulation were initially assessed by two-way analysis of variance (ANOVA). The effects of different concentrations of RDX at each exposure period were then analyzed with one-way ANOVA followed by Duncan's multiple range test (Statistica®, StatSoft, Tulsa, OK, USA). These analyses were performed at the level of significance of α = 0.05. Behavioral observations were qualitatively documented and assessed.

15. Results

15.1. Acute toxicity

15.1.1. Chemical analysis

The HPLC procedure yielded an excellent linearity over the range of RDX standard concentrations with a correlation coefficient of 1.00. The retention time for RDX was approximately 5.4 min. RDX is not highly soluble in water and its highest solubility in the zebrafish water was estimated to be 42 mg L-1 at 28°C. The measured concentration of RDX (mean ± SE, *n* = 5) in the treatment groups of the first test (*Definitive test 1*) were 0 ± 0, 10.44 ± 0.20, 13.27 ± 0.05, 16.52 ± 0.05, 20.56 ± 0.06, 25.93 ± 0.11, 32.08 ± 0.03 and 39.88 ± 0.09 mg L-1; and in *Definitive test 2* were 0 ± 0, 9.75 ± 0.34, 12.84 ± 0.34, 15.32 ± 0.30, 19.09 ± 0.23, 23.98 ± 0.31, 29.89 ± 29 and 37.12 ± 0.31 mg L-1.

15.1.2. Acute toxicity of RDX

Concentration-dependent mortality was observed during the course of the 96-h acute toxicity tests (Fig. 1). No mortality was observed at any RDX concentration within

the first 24 h of exposure in both definitive tests. The first mortality occurred after 48 h of exposure in both definitive tests, and the incidence at the highest RDX concentration after 96 h reached 100 percent in *Definitive Test 1* and 83 percent in the *Definitive Test 2*. No mortality occurred in the control beakers. The 96-h LC50 was estimated to be 22.98 mg L-1 (CL 21.45- 24.64 mg L-1) and 25.64 mg L-1 (CL 17.06-38.89 mg L-1) for the *Definitive Test 1* and *Definitive Test 2* respectively. The NOEC values on lethality of RDX were 13.27 ± 0.05 and 15.32 ± 0.30 mg L -1 and LOEC values were 16.52 ± 0.05 and 19.09 ± 0.23 mg L-1, in *Definitive Test 1* and *Definitive Test 2* respectively ($p < 0.05$).

15.1.3. Vertebral column deformities

There was a concentration-dependent increase in the overall incidence of vertebral column deformities at the completion of the 96-h exposure to RDX. Kyphosis (Silverstone and Hammell, 2002) was the most frequently observed deformity (Fig. 2). A strong positive correlation ($r = 0.80$) was observed between the RDX concentration and percent of vertebral deformities. The 96-h EC50 was estimated to be 20.84 mg L-1 (Fig. 1), with 95 percent confidence limit of 18.73-23.19 mg L-1. The NOEC and LOEC of RDX for vertebral deformities were 9.75 ± 0.34 and 12.84 ± 0.34 mg L-1, respectively ($p < 0.05$; Fig. 3).

15.1.4. Behavioral observations

Experimental units were observed daily and the general behavior of the fish was noted. There were no grossly abnormal behaviors observed within the first 12 h of exposure in any of the treatment groups. At 24 h of exposure, erratic (whirling) movement was conspicuous at RDX concentrations of ≥ 19.09 mg L-1. After 72 h, most larvae at RDX concentrations of ≥ 23.98 mg L-1 had lost their ability to react when chased with a probe and had become lethargic, lying on the bottom of the beaker with only intermittent periods of swimming activity. There were no obvious behavioral alterations in fish from the control beakers or the three lowest treatment groups (≤15.32 mg L-1) during the exposure.

15.2. Subacute toxicity

15.2.1. Chemicals in the exposure media

The measured concentrations of RDX in treatment tanks were close to the nominal concentrations and they were stable for at least a period of one week in the experimental tanks. The measured concentrations were 0, 1 and 9.6 mg L-1 for the target (nominal) concentrations of 0, 1 and 10 mg L-1 (Fig. 4). RDX was not detected in any of the treatment tanks during the recovery period of the study. MNX was not detected in water of the treatment tanks during the exposures, but it was detected in stock solution. The LC-MS analysis indicated that a 10-mg L-1 RDX stock solution contained approximately 10 μg L-1 of MNX. Other metabolites of RDX (TNX, DNX) were undetectable in stock solution.

15.2.2. Effect of RDX on survival and somatic condition

Only the highest concentration of RDX (9.6 mg L-1) caused mortality in the experimental aquaria. Cumulative mortality in this group was 44.8 ± 8.1 percent by the end of the 12-week exposure period, with most mortalities occurring within the first 8

weeks (cumulative 33 percent by 4 weeks and cumulative 43 percent by 8 weeks). There were no mortalities in the 1-ppm group and only one death occurred in the control group.

Control fish did not grow significantly in length or weight between during the period between 4 and 12 weeks of exposure (one-way ANOVA using data from control fish; $p < 0.05$). (The weight and length of fish at the start of the experiment was not taken.) Fish length was not affected by RDX at any time during the exposure period (Fig. 5A; $p > 0.05$). Body weight was affected at 4 weeks of exposure to RDX at both 1 and 9.6 mg L-1 (Fig. 5B; $p < 0.05$); and at 8 weeks only at 9.6 mg L-1 (Fig. 5B). Condition factor was affected only at 4 and 8 weeks of exposure to 9.6 mg L-1 (Fig. 5C; $p < 0.05$). No effects of RDX on somatic condition were observed at 12 weeks of exposure (Fig. 5A-C; $p > 0.05$). Weight, length, and condition factor of fish sampled at the end of the 2-week period in clean water did not vary among treatment groups ($p < 0.05$; data not shown).

15.2.3. Effect of RDX on behavior and reproductive activity

Although food consumption was not quantified in this study, it was clear that RDX at 9.6 mg L-1 affected the response of fish to food presentation (feeding behavior) relative to fish in the control or 1-mg L-1 treatments within the first several weeks of exposure. Fish in the control and 1-mg L-1groups fed aggressively and captured food from the water surface or the water column, whereas fish in the 9.6-mg L-1 treatment tanks were relatively slow to respond to food presentation and typically ate off the bottom of the tank. In addition, fish exposed to RDX at 9.6-mg L-1 did not respond to tapping on the side of aquaria at the time of feeding (congregation behavior in anticipation of feeding) and also showed a higher incidence of erratic movements (hyperactivity). However, these differences in behavior eventually disappeared after approximately 7 weeks of exposure, when fish in the 9.6-mg L-1 aquaria displayed behaviors similar to those of fish from the other groups.

In the control and 1-mg L-1 RDX exposure groups, spawning (chasing) behavior was normally observed near the surface of the biofilters in the morning hours (7 AM to 9 AM) throughout the duration of the exposure. Embryos and larvae were also occasionally observed on the surface of, or in, the biofilters placed within these aquaria. However, spawning behavior was less prominent and embryos or larvae were not observed in the 9.6-mg L-1 exposure group within the first 10 weeks of exposure. After about 10 weeks of exposure, differences in spawning behavior were no longer observed among the treatment groups and embryos/larvae were also occasionally found in the 9.6-mg L-1 aquaria.

15.2.4. RDX and MNX bioaccumulation and elimination

RDX was accumulated in whole-body zebrafish exposed to water borne RDX. There was no RDX detected in fish from the control tanks (Fig. 6A). Accumulation of RDX was concentration- and time-dependent (2-way ANOVA; $p < 0.05$). The BCF was concentration-independent but time dependent; it increased from levels ≤ 1 at 4 and 8 weeks to > 2 at 12 weeks (Fig. 6B). RDX was not detected in the whole-body extract of zebrafish after 2 weeks of rearing only in the zebrafish-water (data not shown).

MNX was also detected in the tissue extracts from RDX-exposed fish but not from control fish. The concentration of MNX was concentration-dependent (Fig. 6C).

Since MNX concentrations in treated-tank water were undetectable, the BCF of MNX could not be determined.

15.2.5. Effect of RDX on gonadal histology

At the completion of the 12-week exposure, all male and female fish examined contained the various stages of germ cell development assessed in this study. Notably, post-ovulatory follicles were observed in several females from all treatment groups, and the percent testicular section area occupied by sperm did not vary among treatments ($P > 0.05$). No gross histopathological features were noted in any of the preparations examined.

15.2.6. Genotoxic effect of RDX in testicular cells

RDX did not cause DNA damage in testicular cells. Comet positive cells were detected in all treatments (approx. 8 percent) including control group (Fig. 7), and we consider this as natural (normal) DNA damage (apoptosis).

16. Discussion

16.1. Acute Toxicity

Although the toxicity of RDX has been relatively well studied in mammals (Hart, 1977; Levine et al., 1981a,b), the information for aquatic organisms is limited to few species. The acute toxicity of RDX has been determined in fathead minnows, bluegill, rainbow trout and channel catfish (Burton et al., 1994; Bentley et al., 1977). Other than the study of the Burton et al., (1994) with fathead minnow, the acute toxicity in most other studies was estimated from the nominal concentrations of RDX either in test water or in DMSO. In the present study with zebrafish we used the measured concentration of RDX to evaluate the LC50, NOEC and LOEC of this compound in larval zebrafish. In fathead minnow, Bentley et al., (1977) reported that the 96-h LC50 of RDX (nominal concentrations) is > 100 mg L-1 for embryos, 43 mg L-1 for 1-h post hatched larvae, 3.8 mg L-1 for 7-day-old larvae, 16 mg L-1 for 30-day-old juveniles, and 11 mg L-1 for 60-day-old fish. Burton et al., (1994) reported that the 96-h LC50 (measured concentration) for 15-17-day-old fathead minnow was 12.7 mg L-1. Similarly, the 96-h LC50 (nominal concentrations) for juvenile bluegill was 6.0-6.4 mg L-1; for fingerling rainbow trout was 6.4 mg L-1; and for juvenile catfish was 4.1-13 mg L-1 (Bentley et al., 1977). In the present study, the 96-h LC50 for 5-day-old larval zebrafish was estimated at 22.98 mg L-1 (95 percent confidence limit of 21.45-24.64 mg L-1). Thus, the acute toxicity of RDX in zebrafish is similar to those reported in other fishes at various stages of development.

It is noteworthy that RDX seems to be more toxic to aquatic vertebrates than aquatic invertebrates (Etnier, 1986). In one study, RDX concentrations at the solubility limit of the compound under specific test conditions were found to be not toxic to daphnia (*Ceriodaphnia dubia*), hydra (*Hydra littoralis*), and midge (*Paratanytarsus parthenogeneticus*) (Peters et al., 1994). Similarly, RDX at concentrations >100 mg L-1 (nominal concentration) was not toxic to the water flea (*Daphnia magna*), scud (*Asellus militaris*) and the midge (*Chironomous tentans*) (Bentley et al., 1977). It is unclear why there is a relatively large difference in RDX toxicity levels between aquatic vertebrates

and invertebrates, but it may be attributed to physiological differences between the taxa or to differences in the respective mechanism of RDX toxicity.

Although RDX is not highly soluble in water, acute toxic effects of this compound in aquatic vertebrates are observed within its solubility range. The origin of RDX in water bodies is mainly waste water discharges from army ammunition plants or run off from contaminated sites (Sunahara et al., 1999). Concentrations of RDX in water have been found to be as high as 109 mg L-1 (Ryon et al., 1984), and at this concentration RDX has been demonstrated to be well above the acute toxic level to aquatic vertebrate species (Bentley et al., 1977; Burton et al., 1994; present study). The toxicity of RDX may also depend on the stage of development of organisms. In fishes, larvae are generally considered to be the more susceptible to contaminant exposure than the adults (Wedemeyer, 1996). Thus, larval toxicity data may be a better indicator of contaminant effects in fishes for the purpose of ecological risk assessment.

Vertebral deformities also showed a concentration-dependent pattern (Fig. 3) with an estimated EC50 value of 20.84 mg L-1. The NOEC and LOEC of RDX for vertebral deformities were 9.75 ± 0.34 and 12.84 ± 0.34 mg L-1, respectively, which are slightly lower than the NOEC and LOEC for lethality (13.27 ± 0.05 mg L-1 and 16.52 ± 0.05 mg L-1, respectively). These values must be considered with some caution, however, since they are based on measurements taken on fish that survived the exposures; and are biased toward the most tolerant individuals of the population. (It is difficult to determine deformities on dead fish because of postmortem artifacts.) However, there was a strong correlation between RDX concentration and the incidence of vertebral deformities. Spinal curvatures have been previously observed in fathead minnow after chronic exposure to RDX, although concentration-dependent effects were not established statistically (Burton et al., 1994). In feral fishes, vertebral column deformities have been reported in individuals inhabiting places contaminated with heavy metals, bleached kraft mill effluent, and chlorinated benzoquinones (Bengtsson, 1975; Bengtsson, 1979 and Halver et al., 1969). In zebrafish, spinal curvatures have been associated with exposure to organophosphate, organochlorine, and carbamate insecticides (Bengtsson 1975; Couch et al., 1977; Incardona et al., 2004) and also with old-age and muscular abnormalities (Gerhard et al., 2002). The mechanism of the vertebral deformities caused by RDX is unknown for fishes. RDX has mutagenic effects in mice and has been classified as a possible human carcinogen (class C) (USEPA, 1994).

Exposure to RDX in mammals causes a variety of central nervous system disorders (Levine et al., 1981a,b), including abnormal behavior that correlates positively with dosage of RDX exposure (MacPhail et al., 1985). Acute toxic effects of RDX have been reported in humans upon ingestion of C-4, which consists mostly of RDX (Stone et al., 1969). The symptoms of acute RDX exposure in this report concerning humans (Stone et al., 1969) included generalized convulsions and unconsciousness, muscular twitching, hyperactive reflexes, headache, temporary amnesia, disorientation and asthenia. In the present study, acute toxic effects RDX on behavior of larval zebrafish included erratic swimming patterns (whirling movement) and, after several days, lethargic behavior. Little information is presently available on the effects of RDX on the behavior of aquatic vertebrates.

The information obtained in this study may be of use in efforts to assess the ecological risk of RDX in the aquatic environment. In this regard, further research is

needed to determine the sublethal effect of RDX on the general health, growth, and reproduction of fishes. In addition, the toxicity of RDX metabolites should also be examined. This study is part of an ongoing larger project to evaluate the ecotoxicological impact of RDX in fishes. The data obtained will also be of use for the design of subacute toxicity tests.

16.2. Sub-acute toxicity

The nominal concentrations of RDX chosen for the present study (1 and 10 mg L-1) are within the range of environmentally relevant concentrations previously reported (Ryon et al., 1984). Also, the highest measured concentration in the present study (9.6 mg L-1) was less than half of the 96-h LC50 value reported for zebrafish larvae (23-25 mg L-1; Mukhi et al., 2005b).

Significant mortality (almost 50 percent) was observed within the first 8 weeks of exposure of adult zebrafish to RDX at 9.6 mg L-1, but no mortalities occurred at 1 mg L-1. Similar observations have been reported with juveniles of fathead minnow (*Pimephales promelas*). For example, Burton et al. (1994) observed significant mortality after a 4-week exposure to RDX at a concentration of 9.83 mg L-1 but not 1.35 mg L-1. Also, Bentley et al. (1997) reported that survival of fathead minnow was not affected at RDX concentrations below 5.8 mg L-1 following a 30-day exposure. In a recent study with juvenile channel catfish (*Ictalurus punctatus*), Belden et al. (2005) reported no incidence of mortality after exposure to RDX at 2 mg L-1 for 7 days. In the present study, we observed that mortality rates in the presence of RDX at 9.6 mg L-1 decreased with time of exposure and became negligible after 8 weeks. This study with zebrafish is the first to report an acclimation to initially-lethal concentrations of RDX following relatively prolonged periods of exposure in fishes.

Patterns of RDX effects similar to those on survival were observed in regards to gross behavior and somatic condition. Normal feeding behavior was acutely suppressed and hyperactivity was acutely induced in fish exposed to RDX at 9.6 mg L-1, and these effects remained evident until about 7 weeks after the initiation of the exposure. Although the present study was not designed to measure actual feeding rates, it is reasonable to suggest that abnormal feeding behavior translated into suppressed feeding rates. In fact, a decrease in feed consumption was reported in northern bobwhite (*Colinus virginianus*) exposed to RDX (in feed; as low as 83 mg/kg) for 14 days (Gogal et al., 2003). The somatic condition of zebrafish was also temporarily affected by RDX exposure. Namely, RDX at 9.6 mg L-1 suppressed body weight and condition factor at 4 and 8 weeks of exposure but not at 12 weeks. This initial suppression in body weight may be at least partly a result of the apparent suppression in food consumption and the hyperactive behavior induced by RDX at the high concentration. Indeed, significant weight loss and neuromuscular dysfunction (lethargy, hypersensitivity, tremors) have been previously observed in the salamander, *Plethodon cinereus* exposed to 5000 mg RDX/kg soil (Johnson et al., 2004). Burton et al. (1994) also observed lower weights in juvenile fathead minnow exposed to RDX at concentrations greater than 2.4 mg L-1 for 4 weeks compared to controls. Curiously, RDX at 1 mg L-1 also affected body weight in the present with zebrafish although only at 4 weeks of exposure. This observation indicates that exposure to the low concentration of RDX had somatic effects that were not apparent from the gross behavioral observations. It is important to note that no mortality was observed at an RDX concentration of 1 mg L-1. Thus, it seems likely that the

behavioral and somatic adaptations observed after 7-8 weeks of exposure to RDX at 9.6 mg L-1 are not solely the result of a biased sampling due to the occurrence of initial mortality at this RDX concentration (which presumably removed the weakest fish from the population). To our knowledge, this is also the first report of the potential for adaptations in sublethal responses to RDX exposure in fishes.

Normal spawning behavior and the occurrence of embryos/larvae in the aquaria (biofilters) were also suppressed by RDX at 9.6 mg L-1, but this phenomenon was also temporary and lasted about 10 weeks. By 12 weeks of exposure, there seemed to be no gross differences in spawning behavior among treatment groups, and embryos/larvae were occasionally observed in all aquaria. Histological observations of the gonads also failed to reveal reproductive effects of RDX at 12 weeks of exposure. However, the methodology used in the present study may not be adequate to detect relatively small but ecologically significant, long-term effects of RDX exposure on reproductive performance. Follow up, functional studies (e.g., reproductive output and gamete quality) are necessary to assess the chronic effects of RDX on fish reproduction.

There was no treatment effect of RDX on the incidence of comet-positive cells in testes of RDX-exposed fish. This observation could be explained either by a lack of genotoxic effects of RDX in the testes, or by an inability of RDX to cross the blood-testis barrier. Liver cells would have been a better tool to study RDX effects on DNA integrity. The results of the present study allow only the conclusion that RDX does not induce apoptosis in testes of zebrafish.

The octanol-water partitioning coefficient of RDX is low (log Kow = 0.87); therefore the expected bioaccumulation potential of this compound is also low. Indeed, the average bioconcentration factor (BCF) for RDX in zebrafish over the 12-week experimental period was 1.4. Short term measurements (< 7 days) of RDX accumulation have been recently conducted in other teleost such as channel catfish (Belden et al., 2005) and sheepshead minnow (Lotufo and Lydy, 2005), where the values reported were 2 and 1.7, respectively. In the present study, MNX was also detected in tissue extracts. It is possible that some of this MNX originates from the small level of contamination of the stock RDX used for the present study (MNX was detected at 1000-fold lower concentrations than RDX). However, to explain its level of accumulation in tissue, one would have to assume a BCF of about 30 (data not shown), which seems to be unlikely. As the present study was not designed for MNX detection in the water samples, calculation of the actual BCF for this compound was not possible. Further study is required for determination of the BCF of MNX in zebrafish.

17. Study Records and Archive

Study Records will be maintained at The Institute of Environmental and Human Health (TIEHH) Archive for a minimum of one year after the completion date of the study.

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19. Figures

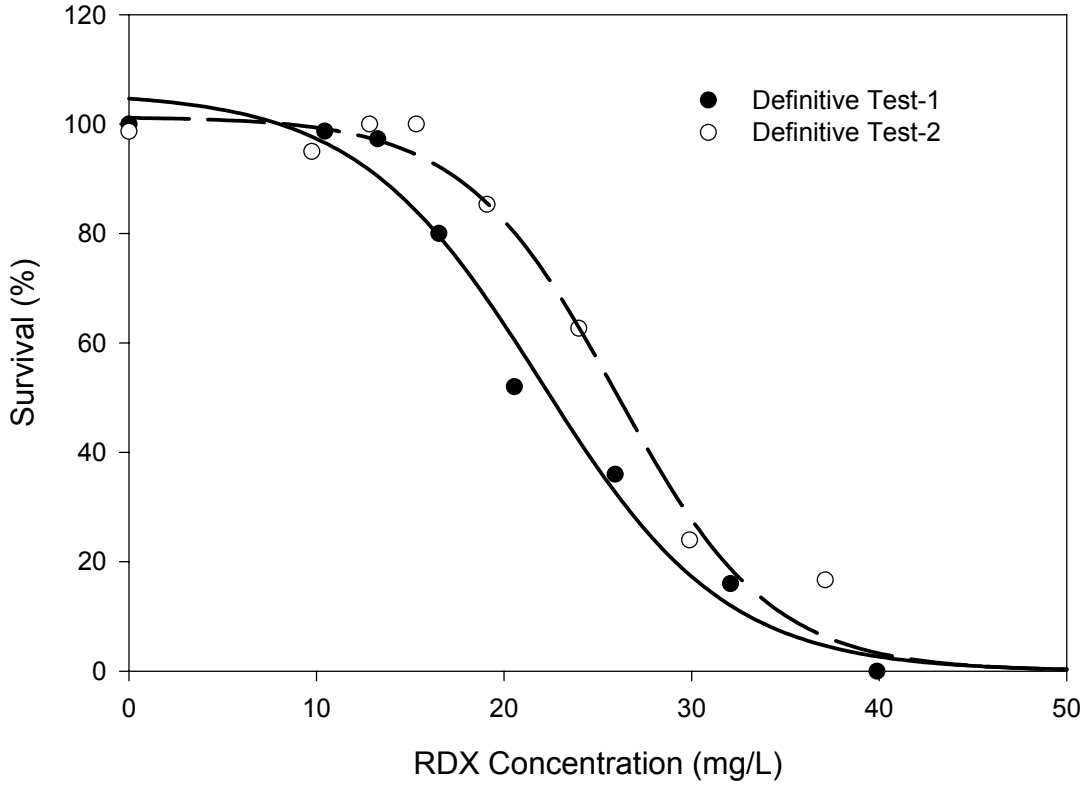


Figure 1. Acute toxic effect of waterborne RDX on larval zebrafish. Survival (mean ± standard error) was determined after 96 h of exposure to various concentrations of RDX in two separate definitive tests. Note that the measured RDX concentrations were slightly different between *Definitive test 1* (● .) and D*efinitive test 2* (○). For each test, the 96-h LC50 and EC50 were estimated by probit analysis from the percent survival after 96 h of exposure.

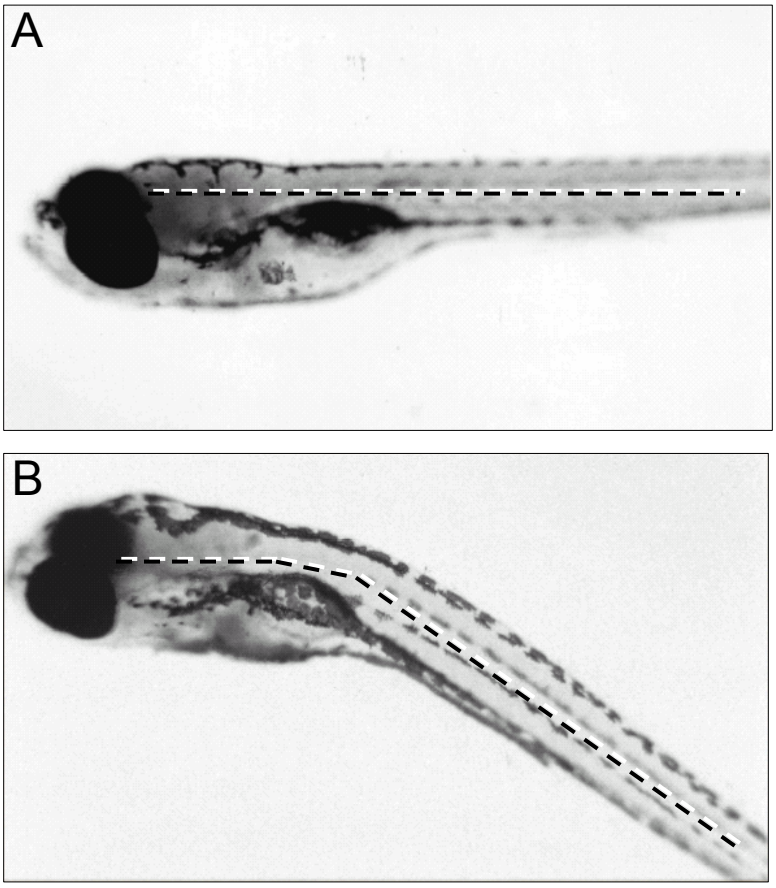


Figure 2. Vertebral column deformities caused by waterborne RDX in larval zebrafish. Control larvae (A) had a straight longitudinal axis running from the head through the trunk to the caudal fin (dashed line). RDX caused different types of vertebral deformities in exposed larvae (B), the most common being kyphosis - a downward curvature of the longitudinal axis (dashed line) in the region over the abdominal cavity.

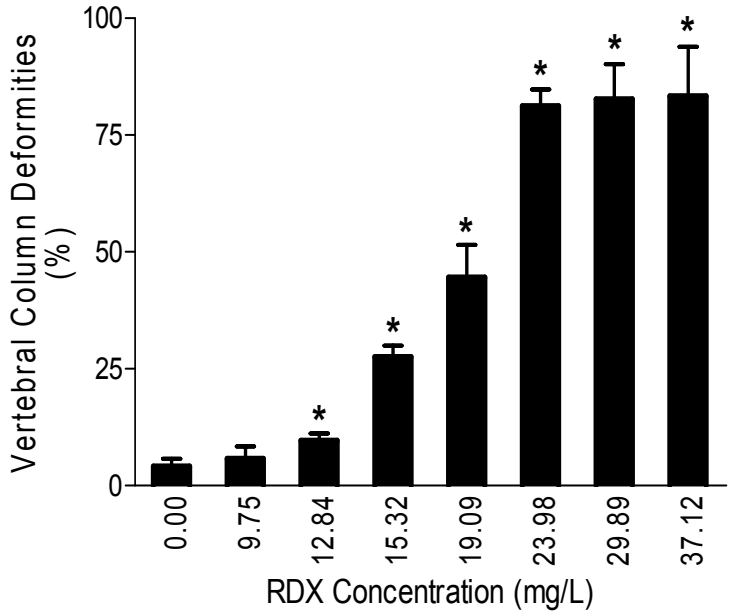


Figure 3. Concentration-dependent effect of waterborne RDX on vertebral deformities (mean ± standard error) in larval zebrafish. The data shown was collected during the *Definitive Test 2* (see text), and includes all types of deformities. There was a significant effect of waterborne RDX concentration on overall vertebral deformities ($p < 0.05$). The treatments that were significantly different from control are indicated by an asterisk (*, $\alpha = 0.05$). The NOEC and LOEC of RDX for vertebral deformities were 9.75 ± 0.34 and 12.84 ± 0.34 mg L-1, respectively ($p < 0.05$).

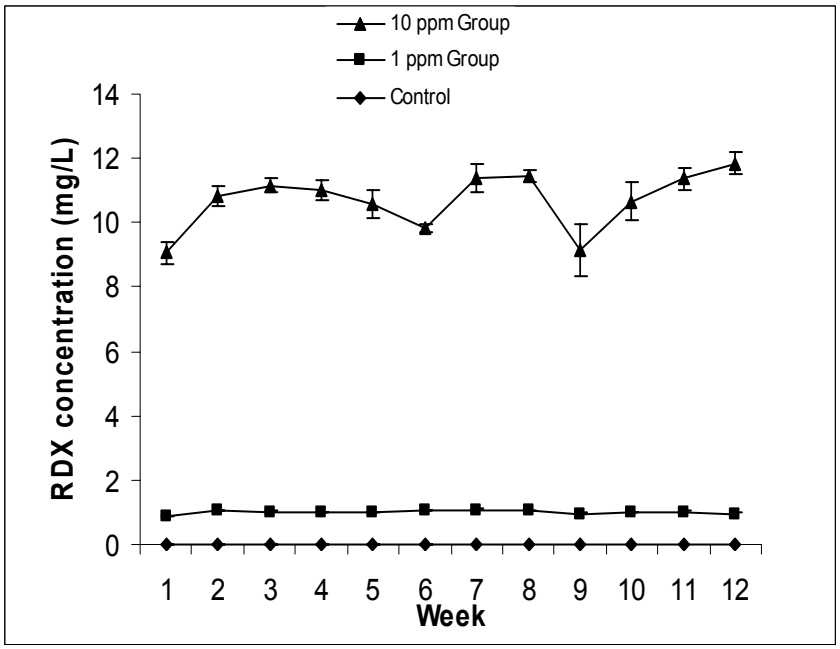
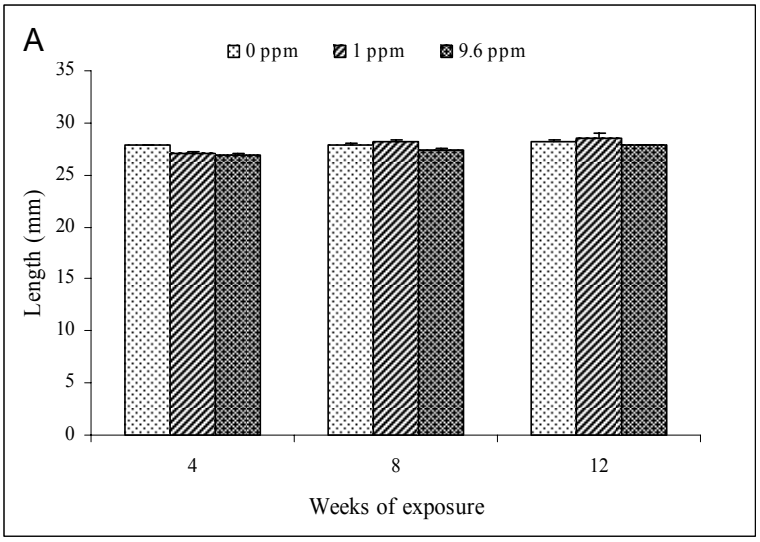
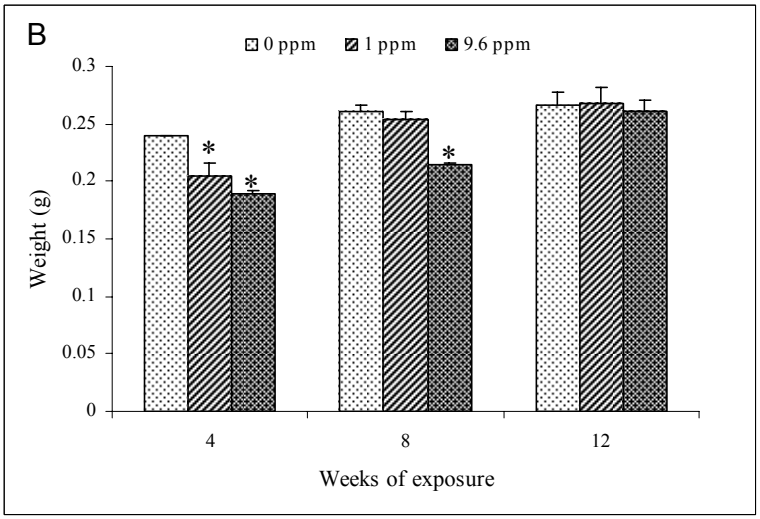


Figure 4. Measured RDX concentration in exposure water during the first 12 weeks of exposure.





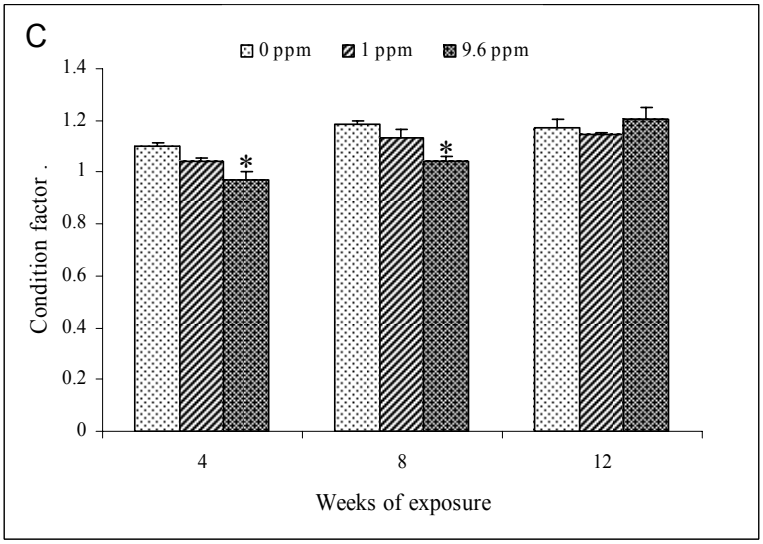


Figure 5. Effects on length (panel A), weight (panel B), and condition factor (panel C) of zebrafish at 4, 8 and 12 weeks of exposure to RDX. Each bar indicates mean ± SE, n =3). Only treatments that were significantly different from control are indicated by an asterisk (*, $\alpha < 0.05$).

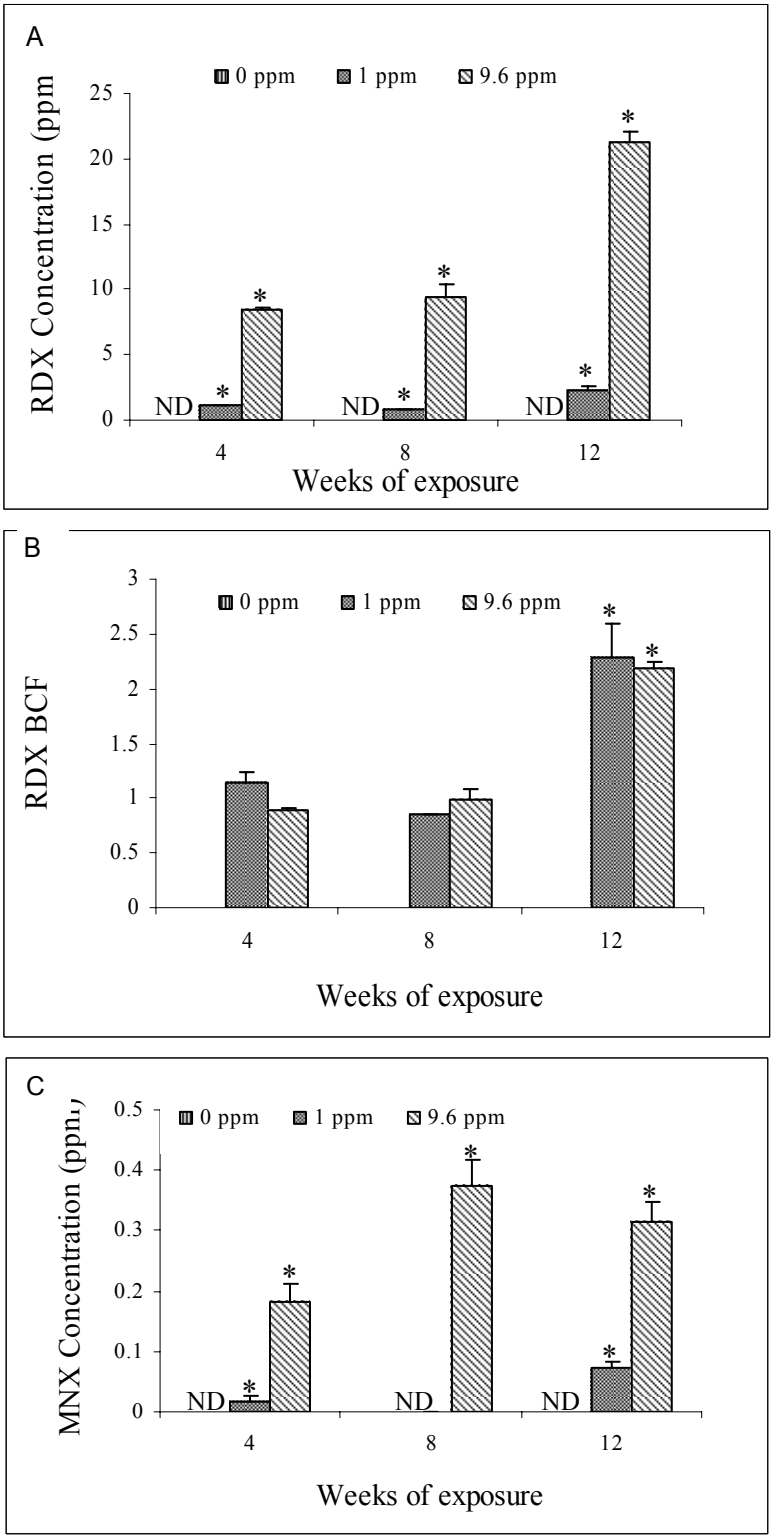


Figure 6: RDX bioaccumulation (panel A) and bioconcentration factor (BCF) of RDX (panel B) and MNX bioaccumulation (panel C) in whole-body of zebrafish at 4, 8 and 12 weeks of exposure. Each bar indicates mean ± SE (n=3) and asterisks (*) indicate that the treatment is significantly different from control. RDX and MNX were not detected (ND) in control fish.

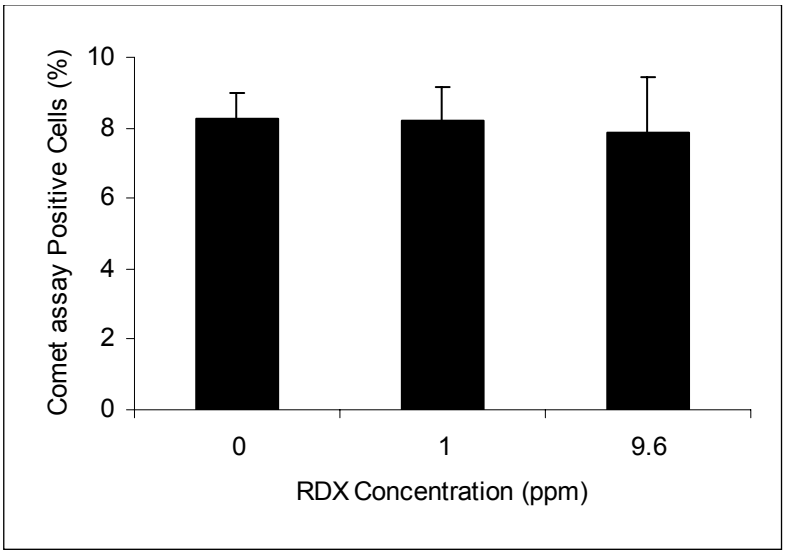


Figure 7: Effect of RDX on the incidence of comet-positive cells in the testis of zebrafish. There was no treatment effect on DNA integrity after 12 weeks of exposure ($p > 0.05$).

20. Appendices

Study Protocol
Changes to Study Documentation Forms

A FINAL REPORT

Biological Availability and Invertebrate Toxicity of Explosive Metabolites in Soil

STUDY NUMBER:	INVTOX-05-01
SPONSOR:	Strategic Environmental and Research Development Program SERDP Program Office 901 North Stuart Street, Suite 303 Arlington, VA 22203
CONTRACT ADMINISTRATOR:	The Institute of Environmental and Human Health Texas Tech University/TTU Health Sciences Center Box 41163 Lubbock, TX 79409-1163
TESTING FACILITY:	The Institute of Environmental and Human Health Texas Tech University Box 41163 Lubbock, TX 79409-1163
TEST SITE:	The Institute of Environmental and Human Health Texas Tech University Box 41163 Lubbock, TX 79409-1163
RESEARCH INITIATION:	January 2004
RESEARCH COMPLETION:	July 2005

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GOOD LABORATORIES PRACTICES STATEMENT

This study was conducted in accordance with established Quality Assurance Program guidelines and in the spirit of the Good Laboratory Practice Standards whenever possible (40 CFR Part 160, August 17, 1989).

Submitted By:

_____ _____

Todd A. Anderson Date

QUALITY ASSURANCE STATEMENT

This study was conducted under The Institute of Environmental and Human Health Quality Assurance Program and whenever possible to meet the spirit of the Good Laboratory Practices as outlined in 40 CFR Part 160, August 17, 1989.

Submitted By:

_____ _____

Ryan Bounds Date:
Quality Assurance Manager

1.0 DESCRIPTIVE STUDY TITLE:
Biological Availability and Invertebrate Toxicity of Explosive Metabolites in Soil

2.0 STUDY NUMBER:
INVTOX-05-01

3.0 SPONSOR:
Strategic Environmental and Research
Development Program
SERDP Program Office
901 North Stuart Street, Suite 303
Arlington, VA 22203

4.0 TESTING FACILITY NAME AND ADDRESS:
The Institute of Environmental and Human Health
Texas Tech University
Box 41163
Lubbock, Texas 79409-1163

5.0 PROPOSED EXPERIMENTAL START & TERMINATION DATES:
Start: 1/1/04
Termination: 7/31/05

6.0 KEY PERSONNEL:
Dr. Todd Anderson, Study Director / Study Advisor
Mr. Baohong Zhang, co-investigator
Ms. Christina Freitag, co-investigator
Mr. Ryan Bounds, Quality Assurance Officer
Dr. Ron Kendall, Principal Investigator

7.0 STUDY OBJECTIVES / PURPOSE:
The proposed focus of the subproject outlined here was to address data gaps related to the potential environmental impact of two degradation metabolites of RDX (TNX and MNX). Specifically, the purpose was to assess the biological availability of TNX and MNX in soil, and to assess the toxicity of TNX and MNX to soil invertebrates.

8.0 STUDY SUMMARY:
The kinetics of uptake of two major of RDX metabolites (MNX and TNX) into passive sampling devices (PSDs), and the ability of PSDs to serve as surrogates for evaluating bioavailability of MNX and TNX were investigated in laboratory sand and two soil types. The results indicate that MNX and TNX absorption into PSDs was best fitted with a polynomial curve model: $y = ax^2 + bx + c$ (y: amount of MNX or TNX absorbed into PSD; x: incubation time of PSDs in soil), with an excellent correlation coefficient (>0.95) for each type of soil amended with 10

mg/kg MNX or TNX. TNX was more readily absorbed by PSDs than MNX. Soil conditions, especially organic matter content, affected MNX and TNX uptake into PSDs. A relatively good correlation between MNX and TNX uptake into PSDs and uptake into earthworms was obtained in two types of natural soils (a silt loam soil from Nebraska and a sandy loam soil from Texas) and laboratory sand. A linear relationship between PSD uptake and earthworm uptake was observed. The correlation coefficients (r^2) were ≥0.82 for all test soils spiked with MNX or TNX. Organic matter content is one major soil factor that affected the ratio of MNX or TNX uptake into earthworms vs. uptake into PSDs. These data indicate that C18 PSDs may be used as a surrogate for soil organisms such as earthworms and provide a simple and easy chemical test for assessing the bioavailability of contaminants in soils.

The acute and chronic toxicity of MNX and TNX to earthworm (*Eisenia fetida*) was evaluated in two types of soil. Results indicated that both MNX and TNX had lethal and sublethal effects on earthworms. Exposure to MNX- or TNX-contaminated soil caused a significant concentration-dependent decrease in survival and growth of earthworms. Earthworms were more sensitive to TNX than to MNX. MNX and TNX were more toxic in sandy loam soil than in silt loam soil, possibly due to higher organic matter content in the silt loam soil. The Lowest Observed Lethal Concentration (LOLC) for both MNX and TNX was 100 mg/kg in the sandy loam soil, and 200 mg/kg in the silt loam soil. No earthworms survived in MNX or TNX-spiked sandy loam soil or silt loam soil at concentrations of 500 mg/kg after 14 days exposure. At 7 days of exposure, the Lowest Observed Effect Concentration (LOEC) for earthworm growth was 50 mg/kg for TNX and 100 mg/kg for MNX in both soil types. After 35 days of exposure, earthworm growth was reduced 8-39% by TNX in sandy loam soil, whereas TNX only inhibited earthworm growth 5-18% at the same concentration range (0.1-100 mg/kg) in silt loam soil. The effect of MNX and TNX occurred early after exposure, usually during the first week. There was no significant difference in earthworm feeding and other behaviors among MNX or TNX concentrations. After 7 days of exposure to MNX and TNX, the LC_{20} and LC_{50} were about 110 mg/kg and 250 mg/kg in sandy loam soil, and 200 mg/kg and 360 mg/kg in silt loam soil. After 14 days of exposure to MNX and TNX, the LC_{20} and LC_{50} were not significantly changed in the sandy loam soil, whereas the LC_{20} and LC_{50} decreased to 96-112 and 216-244 mg/kg in the silt loam soil. After 35 days of exposure, the EC_{20} for MNX was 83 mg/kg in the sandy loam soil, and 151 mg/kg in the silt loam soil. For the same period of exposure, the EC20 for TNX was 92 mg/kg in the sandy loam soil, and 150 in the silt loam soil. The EC_{50}s were 229 and 526 mg/kg for MNX, and 238 and 364 mg/kg for TNX in the sandy loam soil and in the silt loam soil, respectively.

The effect of MNX and TNX on cricket (*Acheta domesticus*) survival and reproductive success was also studied. Our results indicate that RDX metabolites did not have gross adverse effects on adult cricket survival, growth, and egg production. However, MNX and TNX did affect cricket egg hatching; a dose-

response relationship was observed. Tests in contaminated sand indicated that MNX and TNX were toxic. TNX was more toxic to egg hatching than MNX, and developmental stage and exposure time affected cricket hatching. Based on 30 days exposure to MNX or TNX, the ECs for 20, 50, and 95 were 21, 52, and 99 μg/g for MNX, and 12, 48, and 97 μg/g for TNX in sand tests. The lowest observed effect concentration was 10 mg/kg in sand with TNX, whereas it was 100 mg/kg for MNX. No gross abnormalities in cricket nypmhs were observed in all experiments indicting that neither TNX nor MNX is teratogenic in this assay.

9.0 TEST MATERIALS:

Test Material: sandy loam soil (Terry County, TX) and a silt loam soil (Harlan County, NE)

Test Chemical: MNX (hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine)
CAS Number: 5755-27-1
Characterization: Purity confirmed by source.
Source: SRI International

Test Chemical: TNX (hexahydro-1,3,5-trinitroso-1,3,5-triazine)
CAS Number: 13980-04-6
Characterization: Purity confirmed by source.
Source: SRI International

Reference Chemical: acetone
CAS Number: 67-64-1
Characterization: ACS-Certified.
Source: Fisher Scientific

Reference Chemical: acetonitrile
CAS Number: 75-05-8
Characterization: ACS-Certified.
Source: Fisher Scientific

Reference Chemical: deionized water (18MΩ)
CAS Number: NA
Characterization: The quality of the water was confirmed by analytical tests.
Source: Milli-Q

10.0 JUSTIFICATION OF TEST SYSTEM:

Recently, much research effort has been focused on filling data gaps related to the fate/toxicity of explosive materials (EMs) in soil. With the exception of CL-20, the results of this effort have been a better characterization of the bioaccumulation/bioavailability, invertebrate toxicity, and plant toxicity and uptake of a variety of parent EMs and the development of Ecological Soil Screening Level (Eco-SSL) benchmarks for use in ecological risk assessments at explosives-contaminated sites.

While much invertebrate and plant toxicity information has been recently obtained through SERDP-sponsored research on the parent EMs, to our knowledge very little data exist on the potential environmental impact of the degradation metabolites of compounds such as HMX and RDX. The products of the biotic and abiotic degradation of these compounds may also pose toxicological risk to terrestrial and aquatic organisms.

11.0 TEST ANIMALS:

Invertebrates

Cricket (*Acheta domesticus*)
Earthwrom (*Eisenia fetida*)

12.0 PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:

All test systems (earthworms, crickets, PSDs) were placed in bottles with labels containing the appropriate identification information for the test system. Collected samples were placed in individually labeled bags/containers and stored appropriately according to TIEHH SOP IN-3-02.

13.0 EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:

A variety of controls were used throughout the course of the experiments to ensure the quality of the data generated. Solvent controls (soil amended with acetone or acetonitrile only) and negative controls (soil without test compound or solvent) were included in all trials. The solvent controls were prepared in the same manner as toxicant-spiked soils without the toxicant. All data analyses were conducted on measured metabolite concentrations rather than nominal concentrations. Data were processed using standard statistical software (SigmaPlot, Version 8.0, and SigmaStat, Version 2.03, SPSS, Chicago, Illinois, USA).

14.0 METHODS:

14.1 Bioavailability Tests

Passive Sampling Devices (PSDs)

Passive sampling devices (PSDs) were constructed as described previously (Awata et al., 1999; Johnson et al., 1995). Five hundred milligrams of C18 was weighed and placed into a 1-oz polyethylene Whirl-Pak® sampling bag (Nasco, Fort Wilkinson, WI). Nominal film thickness of the bag was 63 to 71 μm. Bags were cut into approximate final dimensions of 5.0 x 5.0 cm and then heat-sealed. The approximate surface area of each bag was 50 cm^2.

Soil Preparation and Soil Characteristics

Laboratory sand (Fisher Scientific Inc.) and two types of natural soils, a sandy loam soil from Texas and a silt loam soil from Nebraska, were used in these experiments in order to study the effect of different soil environmental conditions on bioavailability of MNX and TNX. Silt loam soil was collected near Stamford, Harlan County, NE. Sandy loam soil was collected near Ropesville, Terry

County, TX. The physiochemical properties of the study soils (**Table 14.1.1**) were determined by A&L Midwest Laboratories (Omaha, NE) using standard techniques. Each soil was mixed, air dried, and prepared by sieving through a 2-mm sieve prior to use in experiments.

Silt loam soil was spiked using TNX and MNX in acetone to produce a series of concentrations (0.1, 1, 10, 50, 100, 200, 400, or 500 mg/kg). Sandy loam soil and laboratory sand were only spiked at 10 mg/kg MNX or TNX. Each toxicant was tested separately. Spiked soil samples were thoroughly mixed to distribute test compounds evenly in soil and allow the solvent to evaporate. The spiked soil samples were left for 24 hours in the dark under a chemical hood to permit complete evaporation of acetone. Evaporated soils were hydrated to 75% of their water holding capacity just before the start of the experiments; hydrating the soil with water allowed for further mixing of the test compounds and ensured an even distribution in the soil. The concentrations of MNX and TNX in test soils were measured using GC analysis as described below.

MNX and TNX Uptake from Soil into PSDs
At the beginning of each study, a subsample (125 g) of each MNX- or TNX-spiked soil type was transferred into triplicate 500-mL glass jars followed by a PSD in each jar. Then, another 125 g of the same soil was put into each jar to cover the PSD completely. Finally, the sample jars were sealed with a Teflon-lined lid. Tested jars were kept in the dark at room temperature (20-22 ºC) until sampling (up to 60 days). PSDs were collected from triplicate jars on Days 2, 4, 8, 16, 24, 32, 48, and 60. Collected PSDs were individually transferred to a labeled clean jar and frozen at -20 ºC until extraction.

MNX and TNX Uptake from Soil into Earthworms
A procedure similar to that of the PSD uptake test described above was used for the MNX and TNX uptake experiment from soil into earthworms. A subsample (250 g) of each MNX- or TNX-spiked soil type was transferred into triplicate 500-mL glass jars followed by placing 6 active earthworms (approximate total weight = 2g) in each jar at the beginning of each study. Then, the glass jars were loosely covered with a Teflon-lined lid. Tested jars were kept in the dark at room temperature (20-22 ºC) until sampling (up to 60 days). The lids were removed periodically throughout the experiment to allow air exchange. Earthworm behavior was monitored periodically. After 2, 4, 8, 16, 24, 32, 48, and 60 days of exposure, earthworms were removed from triplicate glass jars and placed on wet filter paper for 24 h to depurate the gut contents, followed by cleaning with distilled water to remove exterior soil particles. Collected earthworms were individually transferred to labeled clean jars and frozen at -20 ºC until extraction.

Sample Extraction
Soil
In order to determine MNX and TNX concentrations in soil at the beginning and certain times during the experiment, three subsamples of each treated soil were

collected and analyzed using the following procedure. Three gram soil samples were thoroughly mixed with 8-10 g anhydrous sodium sulfate (Na_2SO_4). All extractions were conducted using a Dionex ASE 200 extractor (Salt Lake City, UT). Sample-Na_2SO_4 mixtures were loaded into 22-mL cells. Each extraction began with a 5-min preheat, followed by a 5-min static extraction with acetonitrile. The extract was then purged from the cell with nitrogen into a 60-mL glass collection vial. For low concentration soil extracts (0.1 µg/mL), the sample extracts with a volume of 20-22 mL were reduced to 1-2 mL with a rotary evaporator for subsequent Florisil cleanup. The total ASE extraction time was about 20 min per sample.

Five mL extract or concentrated extract from each soil sample was cleaned using a 1 g Florisil column, then filtered through a 0.45 µm Millipore polytetrafluoroethylene membrane into 2-mL vials, and stored (-20 ºC) until analysis.

Earthworms

A procedure similar to that for soil extraction as described above was used to extract MNX and TNX from earthworms. Earthworms were removed from the freezer, weighed, and minced into small pieces. Minced samples were mixed with 5-7 g anhydrous sodium sulfate (Na_2SO_4). Extractions were conducted using a Dionex ASE 200 extractor (Salt Lake City, UT). Five mL extract or concentrated extract from each earthworm sample was cleaned using a 1 g Florisil column, filtered through a 0.45 µm Millipore polytetrafluoroethylene membrane into 2-mL vials, and stored at -20 ºC until analysis.

PSDs

MNX and TNX were extracted from PSD samples in a manner similar to that used for solid phase extraction (SPE). First, the polyethylene sampling bags were opened and the C_{18} sorbent was weighed and recorded. Then, the C_{18} sorbent was individually poured into glass SPE columns. The SPE columns containing the C_{18} sorbent were placed in ports on a vacuum manifold. Next, the sorbent was slowly eluted with 0.5 mL acetonitrile into a 10-mL glass tube. This process was repeated three times. Finally, the extracts were filtered through a 0.45 µm Millipore polytetrafluoroethylene membrane into 2-mL vials, and stored at -20 ºC until analysis.

Chemical Analyses

Concentrations of MNX or TNX in soil, earthworm, or PSD samples were determined by gas chromatography with electron capture detection (GC/ECD). An HP 6890 Series gas chromatograph (GC) equipped with an HP 6890 autosampler and an electron capture detector, all controlled by HP 6890 Series Chemstation from Hewlett-Packard (Agilent, Palo Alto, California, USA), were used. A 30 m x 0.55 mm HP-5 column from Hewlett-Packard (Wilmington, DE, USA) was used for analyte separation. Helium served as carrier gas at a constant flow-rate during the run (80 cm/sec). The oven temperature program began at 90 ºC (2 min hold), increased to 130 ºC at a rate of 25 ºC /min, increased at 10 ºC /min to 200 ºC, and finally increased to 250□C at a rate of 25 ºC /min. The temperature of the injection port was 170 ºC, while that of the detector was 270 ºC. A 2 µL standard or sample was injected in the splitless mode, and the ECD was operated at a constant current. A six-point standard curve was constructed from constant volume (2 µL) injections of calibration standards. Computer-generated peak areas were used to measure sample concentrations. Detection limits were 1 µg/kg for the soil, earthworm, and PSD samples.

14.2 Earthworm Tests

Soil preparation and soil characteristics

Two types of natural soils, a sandy loam soil (Terry County, TX) and a silt loam soil (Harlan County, NE), were selected for this experiment in order to study the potential effects of different soil conditions. The physiochemical properties of the test soils were determined by A&L Midwest Laboratories (Omaha, NE) using standard techniques. The silt loam soil had the following characteristics: 34%

sand, 54% silt, and 12% clay; 2.5% organic matter, and the soil pH was 7.0. The sandy loam soil had the following characteristics: 74% sand, 10% silt, and 16% clay; 1.3% organic matter, and the soil pH was 8.3. Each soil was mixed, air dried, and sieved (2 mm) prior to use in experiments.
Both soil types were spiked using TNX or MNX in acetone to obtain final soil concentrations of 0.1, 1, 10, 50, 100, 200, 400, and 500 mg/kg. Each toxicant was tested separately. Spiked soil samples were thoroughly mixed in order to distribute the contaminant evenly in soil and allow the solvent to evaporate. Spiked soil samples were stored for 24 hours in the dark under a chemical hood to permit the complete evaporation of acetone. Soils were hydrated to 75% of their water holding capacity prior to the start of the experiments; during the addition of water, soil samples were further mixed. Finally, spiked soils (250 g) were put into 500-mL glass jars. All experiments were conducted in triplicate.

A solvent control (soil amended with acetone only) and negative control (soil without test compound or solvent) were included in all trials. The solvent controls were prepared in the same manner as toxicant-spiked soils without the toxicant. The nominal concentrations of MNX and TNX were verified using GC analysis as described below.

Earthworms and toxicity test
The oligochaete red earthworms (*Eisenia fetida*) were obtained from Rays Worms and More (Raytown, MO, USA), and were maintained in earthworm bedding frequently supplemented with peat moss and water at room temperature (about 20 ºC). Only mature adult earthworms having a well-developed clitellum were used in the following toxicity experiments.

Ten adult earthworms were selected, washed, weighed, and placed into replicate glass jars containing the test soil and 0, 0.1, 1, 10, 50, 100, 200, 400 or 500 mg/kg MNX or TNX. The jars were covered with Teflon-lined lids. After 7, 14, 21, 28, and 35 days of exposure, live earthworms were counted, rinsed with deionized water, and then weighed. At the same time, soil samples were collected from each jar and were immediately frozen (-20 ºC) until extraction and GC analysis.

Effect of MNX and TNX on feeding behavior of earthworm
After 35 days of exposure, earthworms were isolated from spiked soil, rinsed with deionized water, and weighed. Earthworms were depurated for 24 hours, rinsed again with deionized water, and then weighed to obtain a final mass. The difference in weights over 24 hours was considered as the feed weight for the earthworm.

Chemical analyses
The chemical analyses for the earthworms followed those described previously in **Section 14.1**.

14.3 Cricket Tests

Crickets
Crickets (*Acheta domesticus*) were purchased from Carolina Biological Supply Company (Burlington, NC). They were fed a diet consisting of dog food and potato. Water was supplied daily. Crickets were maintained in aquaria on a 12 h light:12 h dark photoperiod at room temperature (~20°C). Each aquaria hosted about 15-20 pairs of adult crickets.

Cricket egg production
A total 150 g of fine colored sand (Activa Products Inc., Marshall, TX) was weighed and put into a 200-mL beaker. Then, 20 mL ultra-pure water was used to wet the sand. Sand was evenly divided into 10 groups and put into 10 individual 50-mm diameter Petri dishes (VWR International, West Chester, PA). Each Petri dish contained 15 g wet sand. Finally, the sand-filled Petri dishes were put into a 500-mL Redi-Pak straight-sided jar (VWR International, West Chester, PA). Then, two adult crickets were put into the jar and placed in an incubator overnight at 28 °C in the dark. After about 12 h of incubation, eggs were harvested.

Effect of RDX metabolites on adult cricket survival and egg production
Acclimated adult crickets were fed MNX- or TNX-contaminated dog food for 2 weeks. Contaminated food was prepared according to the following procedure. Twenty grams of dog food was spiked with 1000 µg/mL MNX or TNX in acetonitrile to obtain final food concentrations of 0, 10, and 100 µg/g. MNX and TNX were spiked individually. Spiked food samples were thoroughly mixed in order to distribute the contaminant evenly in food and allow the solvent to evaporate. Spiked food samples were stored for 24 hours in the dark under a chemical hood to permit the complete evaporation of acetonitrile. Food and water were supplied daily. Each treated group (40 adult crickets) was housed in one individual aquarium. The behavior and survival of crickets was observed daily. After 2 weeks of treatment, the number of surviving crickets was recorded. Then, they were allowed to lay eggs in clean colored sand following the same procedure described above.
All eggs were maintained in the sand and an incubator at 28 °C in the dark. After 45 days, the number of eggs and nymphs were determined.

Sand test: effect of MNX and TNX on cricket egg hatching
Two healthy adult crickets were put into a 500-mL glass jar. Each jar contained a 50-mm diameter Petri dish in which 15 g of MNX or TNX-contaminated sand was contained. Contaminated sand was prepared according to the following procedure. First, 75 g sand was weighed for each treatment. Then, the sand was spiked with 1000 µg/mL MNX or TNX in acetonitrile to final concentrations of 10 or 100 µg/g, individually. Spiked sand was thoroughly mixed in order to distribute the contaminant evenly and allow the solvent to evaporate. Spiked sand was stored for 24 hours in the dark under a chemical hood to permit the complete evaporation of acetonitrile. After 24 hours, the sand was wetted with 10 mL ultra-pure water (> 18 MΩ). Finally, the spiked sand was evenly divided into 5 50-mm

diameter Petri dishes (5 replicates). Another 75 g of sand, as control, was treated using the same procedure except that it was spiked only with acetonitrile.

Crickets were kept in the jars overnight (~12 hours), then removed to allow the eggs laid in the sand to incubate in the presence of MNX or TNX. After 45 days, the number of eggs and nymphs were counted.

15.0 RESULTS:

15.1 Bioavailability Tests

Uptake Kinetics of MNX and TNX into PSDs

To investigate uptake kinetics into PSDs, MNX and TNX were amended into two types of natural soils (silt loam soil and sandy loam soil) and laboratory sand. The amount of MNX and TNX absorbed into PSDs was measured at pre-determined times. The results show both MNX and TNX quickly accumulated into PSDs initially, followed by a slower accumulation phase (**Figure 15.1.1**). The absorption was best fitted using a polynomial curve model ($y = ax2 + bx + c$), with an excellent correlation coefficient (> 0.95) for each type of soil amended with MNX or TNX (**Table 15.1.1**).

Initially, the MNX and TNX uptake rate into PSDs was low, but increased with time. After 4-8 days of PSD incubation, the uptake rate gradually decreased (**Figure 15.1.1**). In sandy loam soil, the TNX uptake rate into PSDs was 61.62 ± 4.36 ng/g/day, then increased to 72.55 ± 9.55 ng/g/day. At 8 days of PSD incubation, TNX uptake decreased, eventually reaching 39.65 ± 4.92 ng/g/day at 60 days of incubation. MNX had a similar uptake rate trend as TNX in sandy loam soil. Both TNX and MNX had a similar uptake rate trend in silt loam soil and laboratory sand (**Figure 15.1.1**). This can be explained by simple chemical diffusion. Initially, MNX and TNX need to diffuse to the sorbent (C_{18}) and overcome the barrier of the PSD membrane. This process takes time. After this initial period, MNX and TNX diffuse into the PSD at a high rate due to the large concentration gradient. As more MNX and TNX diffuse into the PSD, the concentration gradient between the two sides of PSD membrane decreases, so the diffusion rate decreases.

Chemical and physical characteristics of both soil types affected MNX and TNX uptake by PSDs. This is illustrated by the total MNX and TNX absorbed and the uptake rate into PSDs (**Figure 15.1.1**). TNX was more easily absorbed into PSDs than MNX in both types of soil. This may be because the TNX is more polar and volatile, and is easily absorbed by the sorbent (C_{18}). After about 30 days of sampling in MNX- or TNX-spiked soil (10 mg/kg), 1.253 ± 0.279, 1.554 ± 0.011, and 11.918 ± 0.574 μg TNX was absorbed into PSDs in silt loam soil, sandy loam soil, and laboratory sand, respectively. In contrast, 0.939 ± 0.010 and 1.461 ± 0.061 μg MNX was absorbed into PSDs in silt loam soil, and sandy loam soil, respectively. MNX and TNX were easily absorbed into PSDs in laboratory sand, followed by sandy loam soil and silt loam soil. This is likely due to differences in organic matter content. Compared with sand or sandy loam soil, the silt loam soil

contained nearly twice as much organic matter. Organic matter absorbs MNX and TNX, lowering the availability of these compounds in soil.

Chemical and physical characteristics of both soil types and chemicals not only affected the amount and rate of MNX and TNX uptake into PSDs, but also affected the concentration gradient between the inside and outside of the PSD membrane. At the same tested concentration (10 mg/kg), TNX uptake into PSDs was 119.19 ± 5.74% of sand concentration at 32 days of incubation. At a similar time, only 15.54 ± 0.11% and 10.79 ± 0.01% of the soil concentration were found in the PSD for sandy loam and silt loam soil, respectively. Similar results were observed for MNX. Sand does not contain organic matter, which would competitively absorbed MNX and TNX. In addition, the sand has greater porosity than the soils which would facilitate movement of TNX and MNX from sand into the PSD.

Effect of MNX and TNX Concentration on Uptake into PSDs
The higher the concentration of MNX or TNX in soil, the higher the uptake concentration in PSDs. There was an excellent relationship between uptake concentration into PSDs and soil concentration. This relationship was best fitted using a linear model: $y = ax + b$. In silt loam soil, the equation was $y = 0.0673x - 0.0190$ and $y = 0.1338x - 0.4702$ for MNX and TNX, respectively, with correlation coefficients of 0.998 and 0.999 (**Figure 15.1.2 A,B**). These data indicate that TNX more easily diffused into PSDs than MNX regardless of concentration. This result is consistent with the kinetic results described earlier.

MNX and TNX Uptake into PSDs versus MNX and TNX Uptake into Earthworms
A similar uptake pattern was observed for MNX and TNX uptake into earthworms. A relatively good correlation between MNX and TNX uptake into PSDs and uptake into earthworms was obtained in both types of amended natural soils and laboratory sand. A linear relationship between PSD uptake and earthworm uptake was observed (**Figure 15.1.3 and Table 15.1.2**). The correlation coefficients were >0.90 in all test soils spiked with 10 mg/kg MNX or TNX, with the exception of TNX in silt loam soil ($r^2 = 0.82$). These data indicate that MNX and TNX uptake into PSDs has a similar pattern as MNX and TNX uptake into earthworms, suggesting that PSDs can be useful as surrogates for evaluating bioavailability of MNX and TNX or other organic compounds in soil.

15.2 Earthworm Tests

Quantification of selected reduced RDX metabolites in spiked soils
The initial measured concentrations of MNX were 0.146 to 390.781 mg/kg in silt loam soil and 0.031 to 470.821 mg/kg in sandy loam soil. The initial measured concentrations of TNX were 0.079 to 404.055 mg/kg in silt loam soil and 0.067 to 472.43 mg/kg in sandy loam soil. Both control soil samples (solvent control and water control) did not contain for MNX and TNX. The measured concentrations of MNX and TNX in the treatments were slightly different than nominal concentrations.

The measured concentrations of MNX and TNX decreased over the 35-day exposure (Data not shown). This phenomenon was observed at all tested concentrations and in both soil types. However, concentration and soil type effects were different. MNX and TNX degraded faster in silt loam soil than in sandy loam soil (Data not shown). This observation is similar to the results of others (LaChance et al., 2004; Robidoux et al., 2004).

Effects of reduced RDX metabolites on earthworm survival in amended natural soils

Evidence for MNX and TNX toxicity to earthworms was a concentration-dependent decrease in adult earthworm survival (**Figure 15.2.1**). Results indicated that both MNX and TNX were lethal to earthworms. After 7 days of exposure, survival of adult earthworms significantly decreased at nominal concentrations greater than 100 mg/kg (**Figure 15.2.1 a**). After 14 days of exposure, the mortality was significantly increased at each nominal concentration greater than 100 mg/kg. Survival was not significantly different among control, solvent control, and nominal concentrations less than 100 mg/kg (**Figure 15.2.1**). After 21, 28, and 35 days of exposure, mortality did not significantly increase at each tested concentration (data not shown). Data also indicated that earthworms were more sensitive to TNX than to MNX at nominal concentrations greater than 100 mg/kg (**Figure 15.2.1**). After 14 days of exposure, 66.6% and 93.4% of earthworms died in the TNX-spiked silt loam soil at nominal concentrations of 200 and 400 mg/kg, respectively. At the same nominal concentrations, MNX only caused 6.7% and 9.1% earthworm mortality in the silt loam soil.

Soil type may affect the lethality of MNX and TNX. In this experiment, earthworms were more sensitive to MNX and TNX in the sandy loam soil than in silt loam soil (**Figure 15.2.1**). In the sandy loam soil, the Lowest Observed Lethal Concentration (LOEC) for both TNX and MNX was 100 mg/kg (nominal). At the LOEC, TNX and MNX caused 6.7% and 3.9% earthworm mortality after 14 days of exposure, respectively. However, we did not observe earthworm mortality for MNX- or TNX- spiked silt loam soil at this same concentration. The LOEC for MNX and TNX was 200 mg/kg (nominal) in the silt loam soil.

Effects of reduced RDX metabolites on earthworm growth in amended natural soils

Additional evidence for MNX and TNX toxicity to earthworms was the inhibition of earthworm growth. This growth inhibition was concentration-dependent. After 7 days of exposure, earthworm growth was reduced by 50 mg/kg TNX or MNX in both soil types (**Figures 15.2.2 – 15.2.5**). Although earthworm growth inhibition was not statistically significant for MNX-spiked sandy loam soil after 7 days of exposure, a significant decrease in growth was observed after 14 days of exposure (**Figure 15.2.3**).

The effect of TNX and MNX in both soil types on adult earthworm growth was indicated by three growth parameters: weight change (body weight after certain period of exposure – body weight at the beginning of experiment), growth index (body weight after certain period of exposure / body weight at the beginning of experiment) and relative growth index (body weight after certain period of exposure / body weight 7 days earlier). Both weight gain and growth index were significantly decreased at nominal TNX and MNX concentrations of 50 mg/kg or greater at all observation periods (**Figures 15.2.2 – 15.2.5**). However, the change in relative growth index was different from the results of weight gain and growth index. Although there was a significant difference among nominal concentrations of 50 mg/kg or greater after 7 days of exposure, usually there was no significant difference among concentrations after 14 days of exposure. These data indicated that the effect of MNX and TNX on earthworm growth occurred shortly after exposure. After 2 weeks of exposure, the negative effect of MNX or TNX was reduced, and some earthworms recovered from MNX or TNX stress.

Both MNX and TNX inhibit earthworm growth. However, the results indicated that earthworms were more sensitive to TNX than to MNX during the first two weeks of exposure although there was no significant difference over 35 days of exposure (**Figures 15.2.2 – 15.2.5**). Earthworms were more sensitive in the sandy loam soil than in the silt loam soil (**Figures 15.2.2 – 15.2.5**). After 35 days of exposure, earthworm growth was reduced 8-39% by TNX in the sandy loam soil (**Figure 15.2.2**), whereas TNX only inhibited earthworm growth 5-18% at the same concentration range (0.1-100 mg/kg) in the silt loam soil (**Figure 15.2.4**). Similar results were observed for MNX-spiked soils (**Figures 15.2.3 and 15.2.5**).

Effects of selected reduced RDX metabolites on earthworm behaviors in amended natural soils

There was no significant abnormal behavior among the tested concentrations of MNX or TNX except that a majority of earthworms moved to the bottom of glass containers at higher concentrations. Earthworms exposed to high (> 50 mg/kg) concentrations of TNX were less active than earthworms exposed to high (> 50 mg/kg) concentrations of MNX. What caused this behavior is unknown. MNX and TNX may have different effects on the earthworm nervous system.

MNX and TNX also affect feeding behavior of earthworms. Intake of soil by earthworms was slightly decreased with increasing MNX or TNX concentrations (**Figure 15.2.6**). This could explain why weight gain and growth index decreased with increasing RDX metabolites in the tested soils (**Figures 15.2.2 – 15.2.5**). **Figure 15.2.6 a** also shows that earthworms processed more silt loam soil than sandy loam soil at the same spiked concentration. However, in time-dependent feed tests, earthworms took in more sandy loam soil than silt loam soil (**Figure 15.2.6 b**). In that experiment, we only tested earthworms in 10 mg/kg MNX- or TNX-spiked soil. Our results showed that 10 mg/kg MNX or TNX has no significant effect on earthworm growth. **Figure 15.2.6 b** also showed that earthworm feeding decreased as the length of MNX or TNX exposure increased.

15.3 Cricket Tests

Effect of MNX and TNX on adult cricket survival and egg production
After 14 days of feeding with MNX-, or TNX-contaminated food, there was no difference in survival of adult crickets between treatment groups (10 or 100 μg/g MNX or TNX) and the control group. In addition, no gross abnormalities in crickets were observed.

Feeding MNX- or TNX-contaminated food did not affect egg production and the hatching of produced eggs (**Figures 15.3.1 and 15.3.2**). About 40% of the eggs hatched, and no gross abnormalities in the nymphs were observed in any of the test groups.
3.2 Sand test: effect of MNX and TNX on egg production and hatching
Crickets laid eggs in both TNX- or MNX-contaminated and uncontaminated (control) sand. Each cricket laid about 120 eggs during an overnight period, and there was no significant difference among the numbers of eggs laid in contaminated sand or control (**Figure 15.3.3**).

Although TNX or MNX in sand did not affect egg production, they did affect the hatching of eggs, and this effect was concentration-dependent (**Figure 15.3.4**). Cricket eggs began hatching after 30-35 days of incubation in sand. For the control group, 43 ± 5.1 % of eggs hatched after 45 days of incubation in sand without MNX or TNX. Adding 10 μg/g of TNX significantly affected egg hatching; only 31 ± 2.7 % of eggs hatched, a decrease of 29% compared with control. However, the same amount of MNX in sand did not significantly reduce cricket egg hatching. 41 ± 28.1 % of eggs hatched in sand with 10 μg/g MNX. At the high concentration (100 μg/g), both MNX and TNX significantly inhibited cricket egg hatching. Only 3.4 ± 3.4 % and 1.6 ± 3.6 % of eggs hatched in TNX- or MNX-contaminated sand (100 μg/g) after 45 days of incubation, respectively. This indicated that TNX inhibited cricket egg hatching more than MNX at the low concentration (10 μg/g); both MNX and TNX inhibited cricket egg hatching at the higher concentration (100 μg/g). The EC_{20}, EC_{50}, and EC_{95} were 21, 52, and 99 μg/g for MNX; they were 12, 48, and 97 μg/g for TNX.

16.0 DISCUSSION

16.1 Bioavailability Tests

Passive sampling is based on free flow of analyte molecules from the sampled medium to a collecting medium according to Fick's first law of diffusion ((Namiesnik et al., 2005)). This free diffusion is dependent on different chemical potentials inside and outside of the PSD membrane. The higher the chemical potential gradient, the higher the rate of free diffusion. Other factors, such as analyte polarity, water solubility, and vapor pressure also affect the free diffusion of analyte from sampled medium into collecting medium.

In this experiment, we chose octadecyl sorbent (C_{18}) as the collecting medium for MNX and TNX based on its physical and chemical characteristics and previous

studies (Awata et al., 1999; Johnson et al., 1995; Zabik et al., 1992). These previous studies demonstrated that octadecyl is a good sorbent for collecting organic contaminants from soils. These experiments further show that octadecyl is a good sorbent for uptake of MNX or TNX from various types of contaminated soils. The amount of MNX or TNX uptake into C_{18} PSDs increased as MNX or TNX concentrations in soils increased.

Initially, MNX and TNX uptake into PSDs approaches linearity, then the uptake rate slows. This observation is similar to those of Zabik et al. (Zabik et al., 1992) and Persson et al. (Persson et al., 2001) for pesticide uptake into C_{18} PSDs from soil and metal uptake into PSDs from water. The MNX and TNX uptake rates decreased with time. Persson and colleagues attributed this phenomena to the chemical potential gradient decrease as more and more analyte molecules move into the PSDs.

Several factors affect the distribution of MNX and TNX in soil and their free diffusion from soil into C_{18} PSDs, such as organic matter content and soil moisture. Organic matter in soil has the ability to absorb MNX and TNX, and effectively decreases the chemical potential gradient between the octadecyl sorbent and soil. The silt loam soil contained more organic matter than the sandy loam soil, whereas laboratory sand did not contain organic matter. Thus, MNX and TNX diffusion was higher in laboratory sand, followed by sandy loam soil, and silt loam soil.

PSDs are sometimes referred to as semi-permeable membrane devices (SPMDs); a majority of PSDs have a semi-permeable membrane. This important characteristic makes PSDs similar to a cell membrane. Thus, PSDs can serve as surrogates of living organisms for evaluating bioavailability of contaminants in soil and water ((Namiesnik et al., 2005)). Various solid-phase extraction (SPE) devices have been used to assess the bioavailability of organic compounds in soils. Good correlation has been observed between chemical uptake into SPEs and chemical uptake into living organisms (Lanno et al., 2004). Tang and Alexander (Tang and Alexander, 1999) and Morrison et al. (Morrison et al., 2000) demonstrated that the uptake of DDT and its metabolites (DDE, DDD) and PAHs into C_{18} membranes had a linear relationship with absorption of these compounds by earthworms.

We observed that MNX and TNX uptake into PSDs was positively correlated with MNX and TNX uptake into earthworms. A linear relationship between uptake into PSDs and into earthworms was determined. In our previous experiments, the concentrations of tested compounds reached a peak, then decreased due to biodegradation in the earthworms (Awata et al., 1999; Awata et al., 2000). In the present experiments, we did not observe a decrease in concentration in the earthworms. However, we found that the MNX and TNX uptake rate into earthworms slowed over time.

Organic matter content is one major soil factor that affected MNX or TNX uptake into earthworms and uptake into PSDs. Laboratory sand does not contain any organic matter; sand particles did not absorb MNX or TNX. The uptake of MNX and TNX into PSDs and earthworms from laboratory sand was almost the same; the ratio of MNX or TNX uptake into PSDs to uptake into earthworms was nearly to 1:1. Silt loam soil contained more organic matter (2.5%) than sandy loam soil (1.3%). Thus, the silt loam soil absorbed more MNX or TNX than sandy loam soil. The availability of MNX or TNX in silt loam soil was lower than in sandy loam soil. This resulted in less MNX or TNX uptake into PSDs in silt loam soil than in sandy loam soil, and produced a higher ratio of MNX or TNX uptake into earthworms to uptake into PSDs. It is also possible that organic matter content in soil may affect the activity of earthworms. Earthworms may be more active in silt loam soil than sandy loam soil, so they process more silt loam soil than sandy loam soil. This resulted in more MNX or TNX absorption into earthworms in silt loam soil than in sandy loam soil.

16.2 Earthworm Tests

The present study evaluated lethal and sublethal toxicities of RDX metabolites (MNX and TNX) in two types of natural soils. The results demonstrated that exposure to MNX- or TNX- contaminated soil caused significant effects on earthworms, as evidenced by concentration-dependent decreases in survival and growth. **Table 16.2.1** summarizes the toxicity endpoints for MNX and TNX in silt loam soil and sandy loam soil. After 7 days of exposure to MNX or TNX, the LC_{20} and LC_{50} were 110 mg/kg and 250 mg/kg in sandy loam soil, and 200 and 360 mg/kg in silt loam soil. After 14 days of exposure to MNX or TNX, the LC_{20} and LC_{50} were 110 mg/kg and 250 mg/kg in sandy loam soil, and 96-112 and 216-244 mg/kg in silt loam soil. TNX LCs (20 or 50) were slightly lower than MNX LCs; this indicates that TNX was more toxic than MNX. MNX and TNX also reduced earthworm growth. After 35 days of exposure, the EC_{20} for MNX was 82.53 mg/kg in sandy loam soil, and 150.75 mg/kg in silt loam soil. For the same period of exposure, the EC_{20} for TNX was 92.42 mg/kg in sandy loam soil, and 150.07 in silt loam soil. The EC_{50}s were 228.89 and 525.75 mg/kg for MNX, and 237.68 and 364.36 mg/kg for TNX, in sandy loam soil and in silt loam soil, respectively (**Table 16.2.1**).

MNX and TNX are products of RDX metabolism by bacteria under anaerobic conditions (Adrian and Arnett, 2004; Hawari et al., 2000a; Hawari et al., 2000b). Many different laboratory and field studies have shown that RDX is toxic to a number of organisms including microorganisms (Juck et al., 2003; Sunahara et al., 1998), plants (Winfield et al., 2004), invertebrates (Kuperman et al., 2003; Robidoux et al., 2004; Robidoux et al., 2002; Robidoux et al., 2000; Simini et al., 2003), vertebrates (Gogal et al., 2003), and humans (ATSDR, 1995). Simini et al (Simini et al., 2003) demonstrated that RDX was highly toxic to earthworm (*E. fetida*) reproduction. However, they did not find that RDX was lethal or reduced growth of earthworms within the concentration range (0-144 mg/kg) they tested. Schafer and Achazi (Schafer and Achazi, 1999) employed two other terrestrial

invertebrates, *Enchytraeus albidis* and *Fosomia candida*, to test the toxicity of RDX. They found that RDX was lethal at very high concentrations (>1000 mg/kg). However, literature on the toxicity of RDX metabolites is scant. In this study, our results indicated that MNX and TNX had lethal and sublethal toxic effects on earthworms, and the effects were greater than those for RDX. This suggests that the toxicity of RDX may partially come from biotransformation products such as MNX and TNX. In the future, when we consider the risk assessment of RDX, we should be cognizant that MNX and TNX are more toxic than the parent compound. Based on the data we obtained in this experiment, MNX and TNX have hazardous effects on soil organisms and may pose a risk to other ecosystem components.

Many factors affect the toxicity of MNX and TNX to earthworms. One important factor is soil physical and chemical characteristics. Organic matter affects the bioavailability of MNX or TNX and in turn their toxicity. Belfroid et al (Belfroid et al., 1996) hypothesized that the bioavailability of nonpolar organic chemicals in soil was determined primarily by soil organic matter content. Thus, the higher the organic matter content, the lower bioavailability. MNX, TNX, and a majority of other energetic compounds are nopolar organic chemicals; their bioavailability should follow this rule. In this experiment, earthworms were more sensitive to MNX or TNX in sandy loam soil than in silt loam soil. This is because the silt loam soil contains more organic matter (2.5%) than the sandy loam soil (1.3%). Previous studies on other explosives also showed similar results (Robidoux et al., 2004; Simini et al., 2003). Simini et al. (2003) reported that the EC20 of RDX was 19.2 mg/kg, whereas Schafer and Achazi (1999) demonstrated that RDX was not toxic up to 1000 mg/kg. This reason for this difference may be that Simini et al. (2003) used Sassafras sandy loam (SSL) soil, while Schafer and Achazi (1999) used an artificial soil. SSL soil contained 1.2% organic matter compared to 10% organic matter in the artificial soil (Simini et al., 2003). Robidoux et al. (2004) reported that the energetic compound CL-20 was not lethal at concentrations of 125 mg/kg or less in the “RacFor2002” soil but was lethal at concentrations of 90.7 mg/kg or greater in the SSL soil, possibly due to the “RacFor2002” soil containing more organic matter than the SSL soil ((Robidoux et al., 2004)). Another reason that toxicants may be less toxic in organic-matter-rich soil than in soil with little organic matter is that there is usually more microbial activity in organic matter-rich soil than marginal soil. Soil microorganisms can play an important role in biotransformation of toxicants.

16.3 Cricket Tests

The effect of two major RDX metabolites (MNX and TNX) on cricket reproductive success was tested. All of the tests indicated that MNX and TNX have somewhat adverse effects on cricket reproductive success, as evidenced by egg hatching (**Table 16.3.1**). In all experiments, TNX inhibited egg hatching more than MNX. This indicates that TNX was more toxic to cricket eggs than MNX. These toxicity results are similar to our results on earthworms. In our previous RDX metabolite toxicity study with earthworms, we found that the

EC_{50}s were 526 mg/kg for MNX, and 364 mg/kg for TNX in the silt loam soil (Zhang et al., 2005). Both the topical test and sand test were good approaches to determine the toxicity of RDX metabolites. These methods are simple and easy to control. Both are suitable for testing other compounds using crickets or other organisms such as earthworms.

Embryos of the cricket (*Acheta domesticus*) were highly sensitive to chemicals and developed gross morphological abnormalities after exposure to a number of complex organic mixtures (Talmage and Walton, 1991; Walton, 1983). In addition, crickets display a critical period of teratogen sensitivity and an ability to metabolize xenobiotics during development (Walton, 1983). After treatment with benz[g]isoquinoline-5,10-dione, benzo[h]quinoline-5,6-dione, or a chemical impurity isolated from commercially purchased acridine, some eggs developed into cricket nymphs with morphological abnormalities including extra compound eyes, extra heads, and distally duplicated legs (Walton, 1981; Walton et al., 1983). Slight structural modifications of the molecules resulted in a loss of teratogenic activity, although embryotoxicity occurred (Walton et al., 1983). In this experiment, although MNX and TNX inhibited cricket egg hatching under certain conditions, no gross abnormalities in cricket nypmhs were observed. This indicates that MNX and TNX are not mutagens or teratogens in this assay.

Developmental stage and exposure time affected cricket hatching. In this experiment, we found that only eggs exposed to MNX or TNX for at least 20 days produced low hatching rates. There was no significant effect between the control group and the treatment groups that were exposed to MNX or TNX only for 0-10 days. This indicates that early development of cricket embryos may be less sensitive to MNX or TNX, or MNX and TNX only produce a biological affect after they accumulate to high concentrations in eggs with time.

17.0 STUDY RECORDS AND ARCHIVE:

Study Records will be maintained at The Institute of Environmental and Human Health (TIEHH) Archive for a minimum of one year after study completion date.

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APPENDIX

Table 14.1.1 Physiochemical properties of soils used in studies on the bioavailability of MNX and TNX.

Soil	Soil type	Sand (%)	Silt (%)	Clay (%)	Organic matter (%)	pH
Nebraska	Silt loam	34	54	12	2.5	7.0
Texas	Sandy loam	74	10	16	1.3	8.3
Sand	Sand	100	0	0	0	7.0

Table 15.1.1. Models for MNX and TNX uptake into PSDs in two types of natural soil and laboratory sand.

Chemical	Soil	Curve model* $y = ax^2 + bx + c$			Correlation coefficient (r^2)	Time range (days)
		a	b	c		
TNX	Sandy loam	-3.3362	57.851	64.434	0.9769	2-60
MNX	Sandy loam	-0.2805	56.888	-108.46	0.9922	2-60
TNX	Silt loam	-1.1429	78.755	-111.19	0.9537	2-36
MNX	Silt loam	-0.04	24.264	98.674	0.9723	2-36
TNX	Sand	-2.9548	468.35	866.39	0.9861	2-60

* y = amount of MNX or TNX taken into PSDs

x = time (days after PSD placed in soil)

Table 15.1.2. The relationship between uptake into PSDs and uptake into earthworms for MNX and TNX in two soil types and laboratory sand.

Chemical	Soil	Curve model* y = ax + b		Correlation coefficient (r^2)	Time range (days)
		a	b		
TNX	Sandy loam	2.906	-0.3795	0.9267	2-60
MNX	Sandy loam	3.3016	-0.4829	0.9170	2-60
TNX	Silt loam	6.1878	-0.3940	0.8170	2-36
MNX	Silt loam	8.1364	-1.0896	0.9045	2-36
TNX	Sand	0.807	-0.9702	0.9534	2-60

* y = amount of MNX or TNX uptake into earthworms at certain times (mg/kg)
x = amount of MNX or TNX uptake into PSDs at certain times (mg/kg)

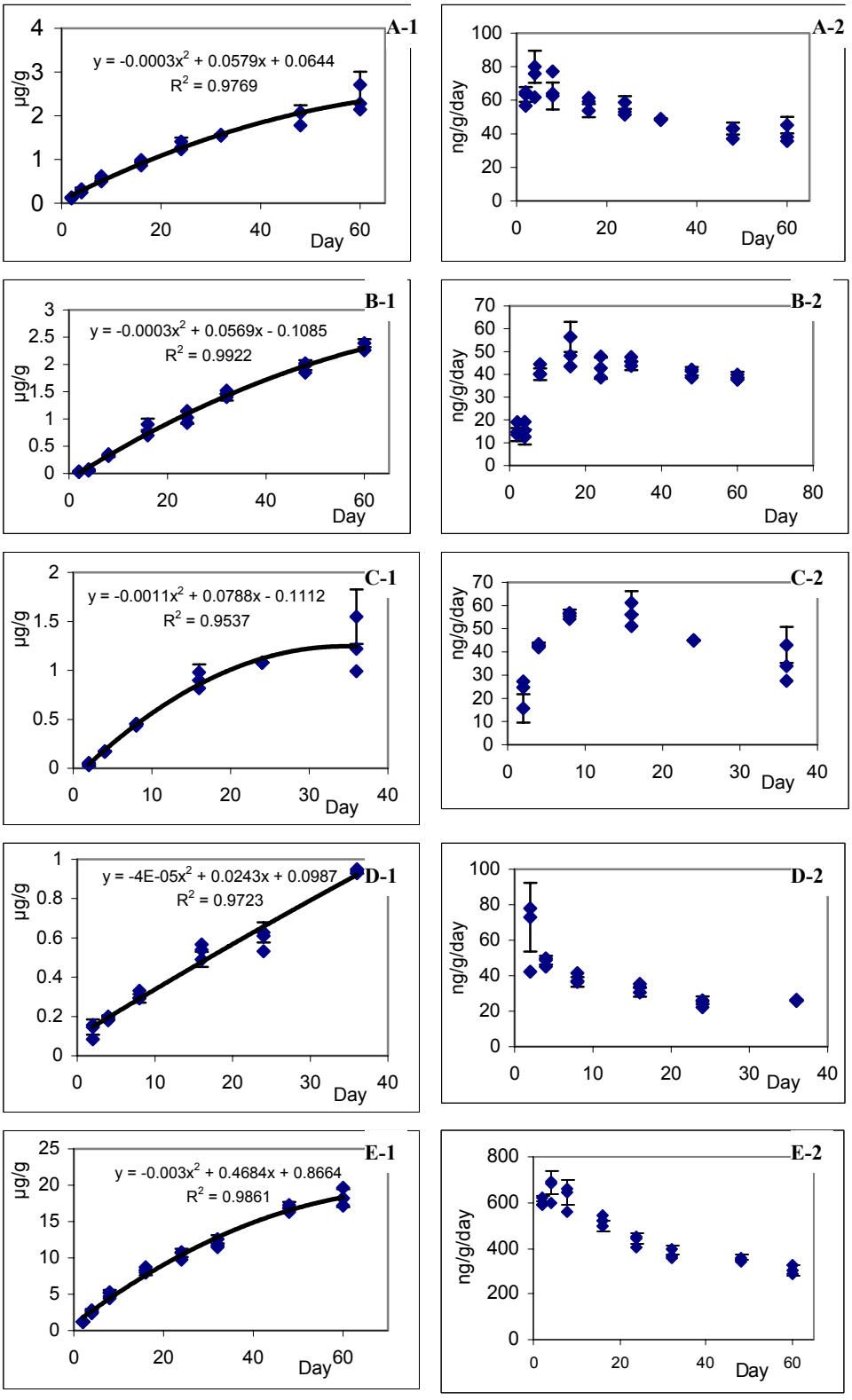

A-1
y = -0.0003x² + 0.0579x + 0.0644
R² = 0.9769
µg/g
Day
A-2
ng/g/day
Day
B-1
y = -0.0003x² + 0.0569x - 0.1085
R² = 0.9922
µg/g
Day
B-2
ng/g/day
Day
C-1
y = -0.0011x² + 0.0788x - 0.1112
R² = 0.9537
µg/g
Day
C-2
ng/g/day
Day
D-1
y = -4E-05x² + 0.0243x + 0.0987
R² = 0.9723
µg/g
Day
D-2
ng/g/day
Day
E-1
y = -0.003x² + 0.4684x + 0.8664
R² = 0.9861
µg/g
Day
E-2
ng/g/day
Day

Figure 15.1.1. MNX and TNX uptake into PSDs in two soils and commercial sand spiked with 10 mg/kg TNX or MNX. A. TNX in sandy loam soil; B. MNX in sandy loam soil; C. TNX in silt loam soil; D. MNX in silt loam soil; E. TNX in sand. 1. Kinetics of MNX or TNX uptake into PSDs; 2. Uptake rate of MNX or TNX into PSDs. Each experiment utilized three replicates. Error bars indicate the standard error of the mean.

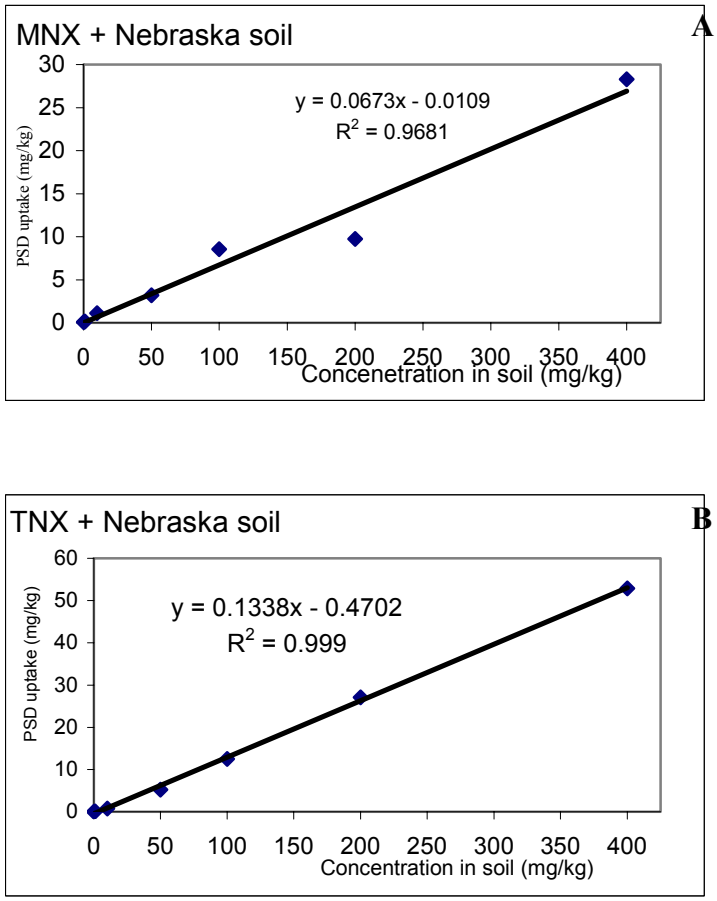
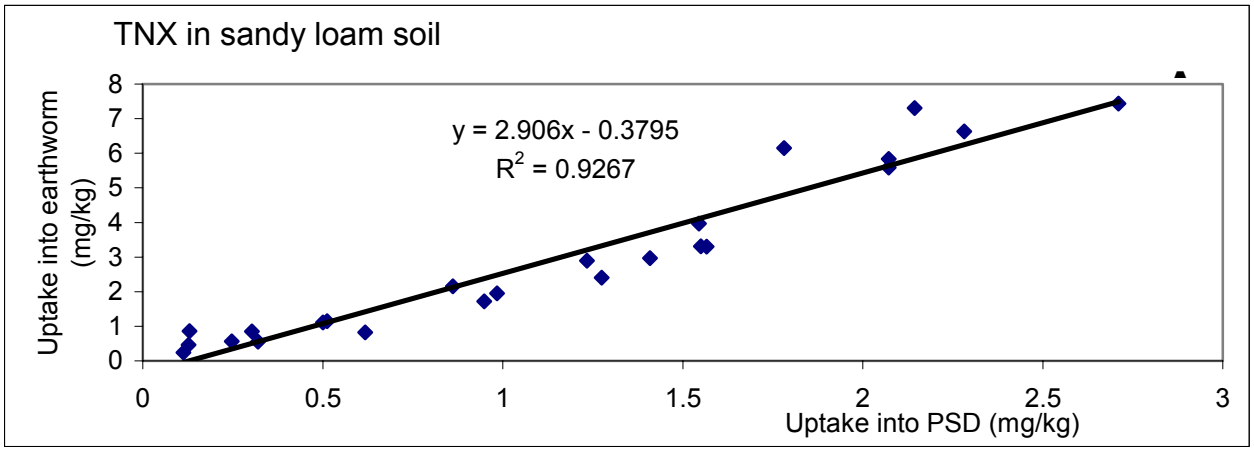
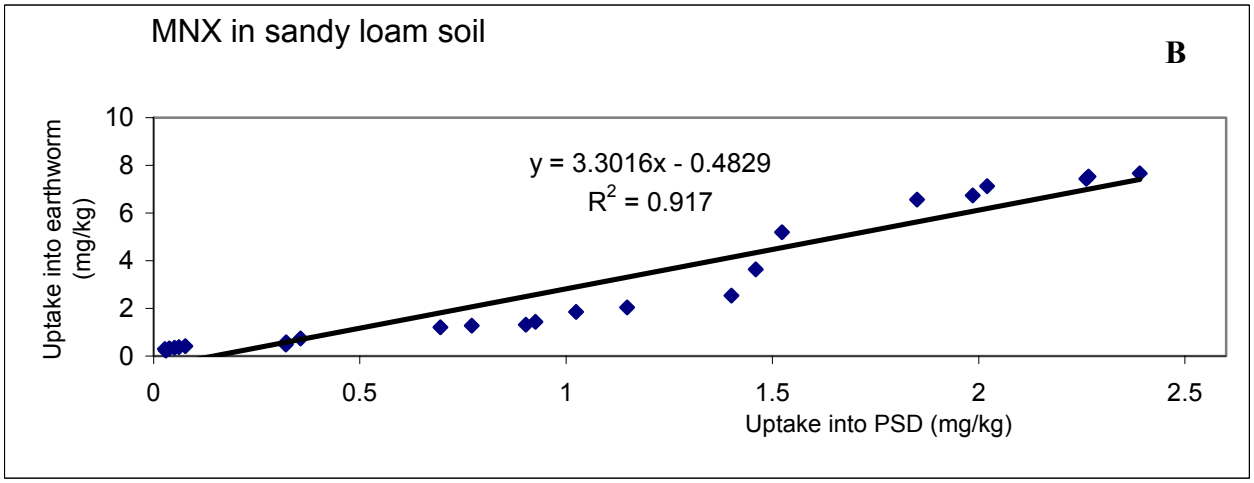
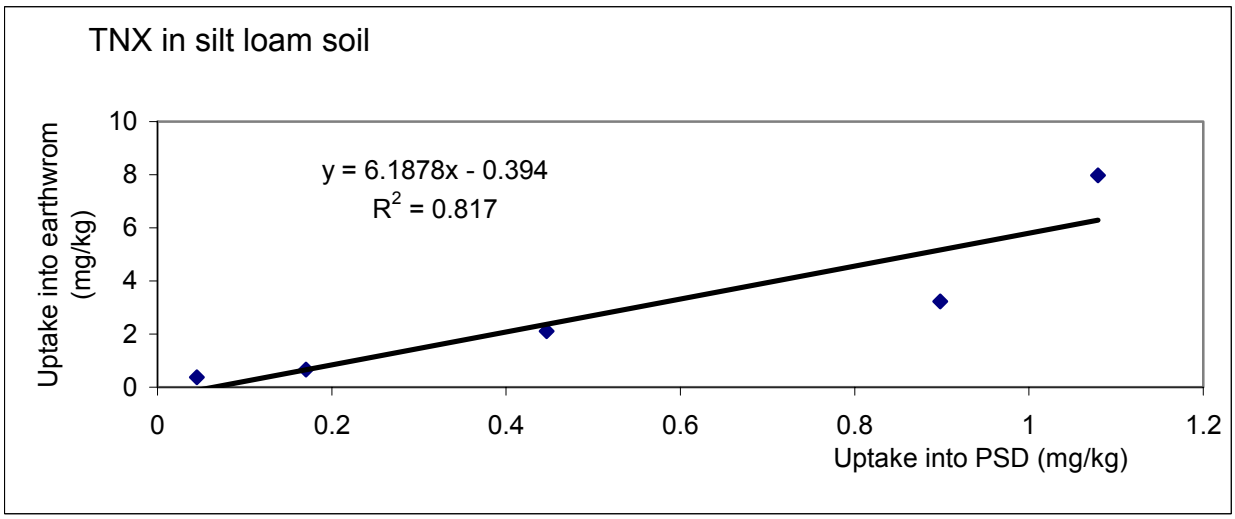
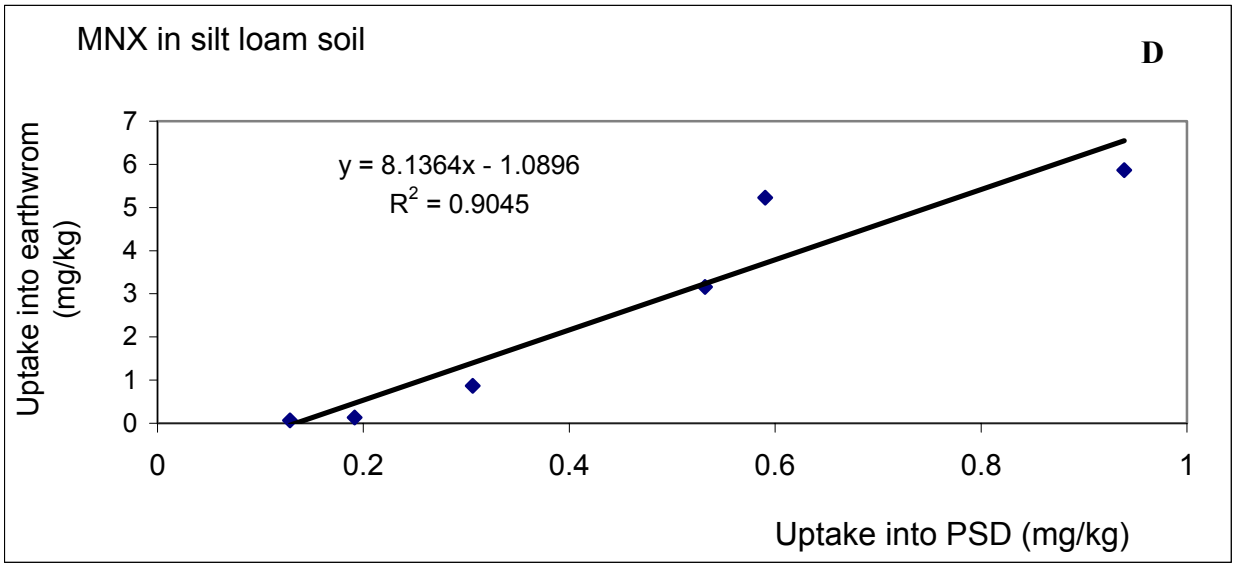


Figure 15.1.2. Effect of MNX and TNX concentrations on MNX or TNX uptake into PSDs in silt loam soil. Total amount of MNX (A) or TNX (B) uptake into PSDs.


TNX in sandy loam soil
y = 2.906x - 0.3795
R2 = 0.9267
Uptake into earthworm (mg/kg)
Uptake into PSD (mg/kg)
0
1
2
3
4
5
6
7
8
0
0.5
1
1.5
2
2.5
3


MNX in sandy loam soil
B
y = 3.3016x - 0.4829
R2 = 0.917
Uptake into earthworm (mg/kg)
Uptake into PSD (mg/kg)
0
2
4
6
8
10
0
0.5
1
1.5
2
2.5


TNX in silt loam soil
y = 6.1878x - 0.394
R2 = 0.817
Uptake into earthwrom (mg/kg)
Uptake into PSD (mg/kg)
0
2
4
6
8
10
0
0.2
0.4
0.6
0.8
1
1.2



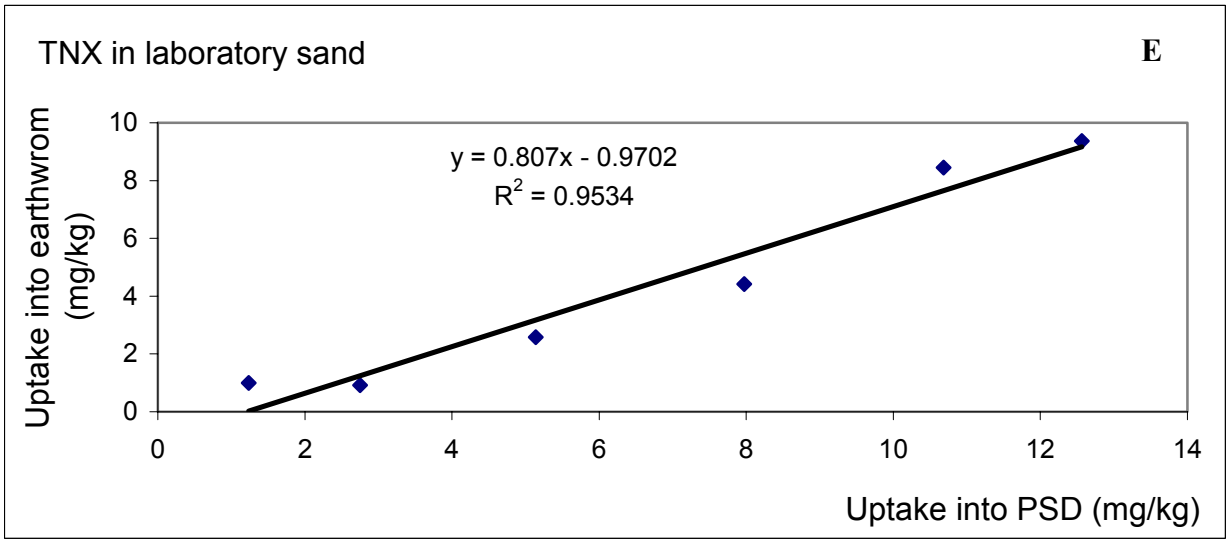
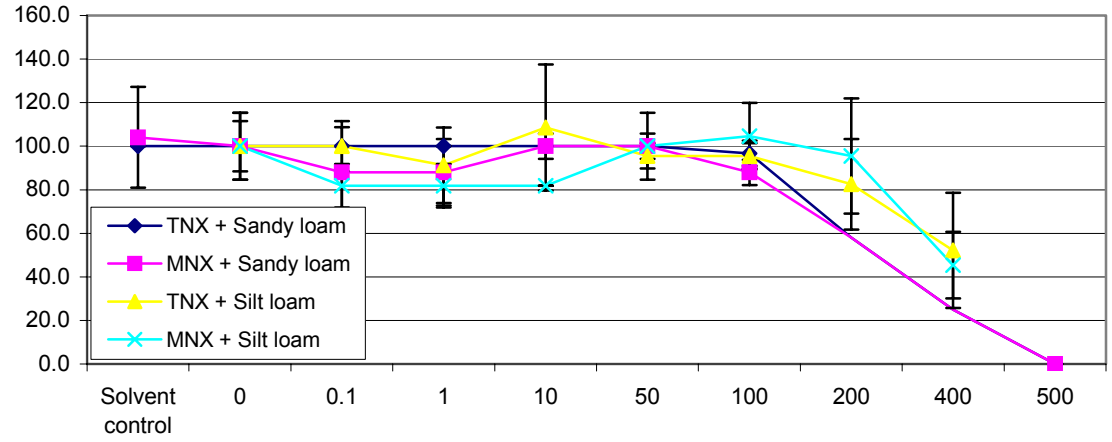


Figure 15.1.3. Correlation of MNX or TNX uptake into PSDs versus MNX or TNX uptake into earthworms in two types of natural soils and commercial sand. **A**. sandy loam soil spiked with 10 mg/kg TNX; **B**. sandy loam soil spiked with 10 mg/kg MNX; **C**. silt loam soil spiked with 10 mg/kg TNX; **D**. silt loam soil spiked with 10 mg/kg MNX; **E**. commercial sand spiked with 10 mg/kg TNX

A. 7 Day



B. 14 day

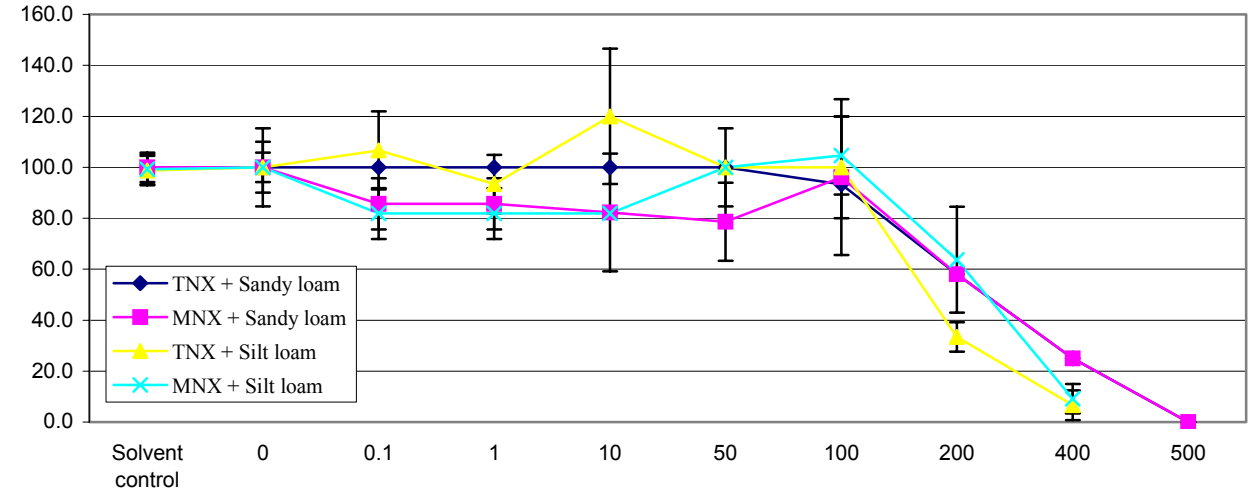
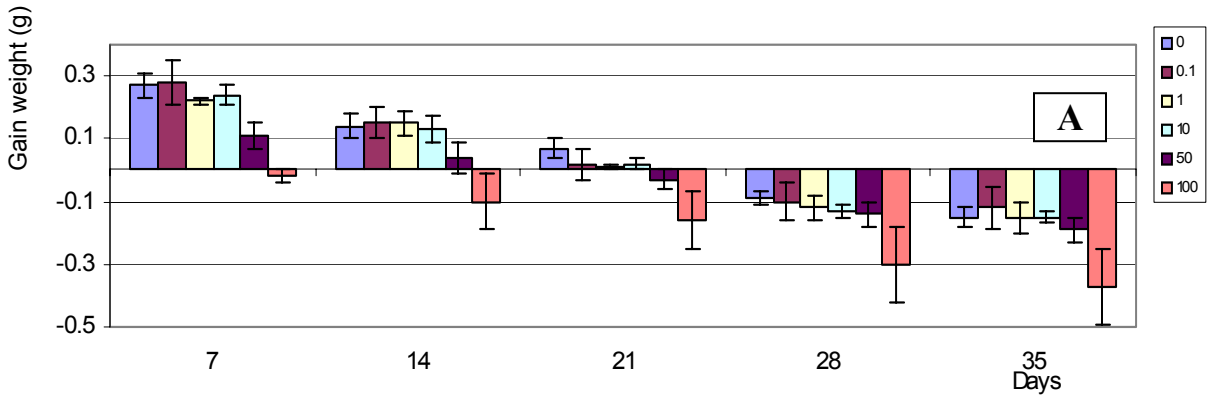
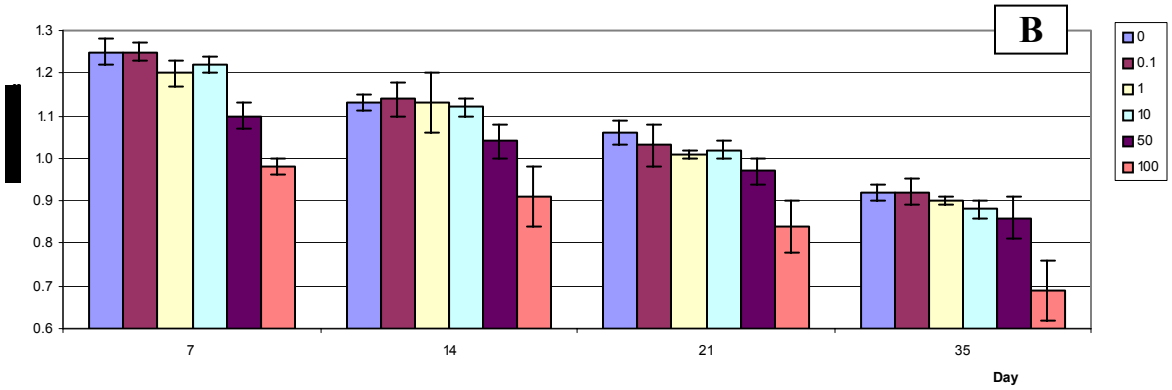


Figure 15.2.1. Lethal effects of MNX or TNX to earthworms in two types of amended natural soils after (**a**) 7 d or (**b**) 14 d of exposure (n = 10 earthworms X 3 replicates / concentration at start of experiments). Error bars show standard deviation.





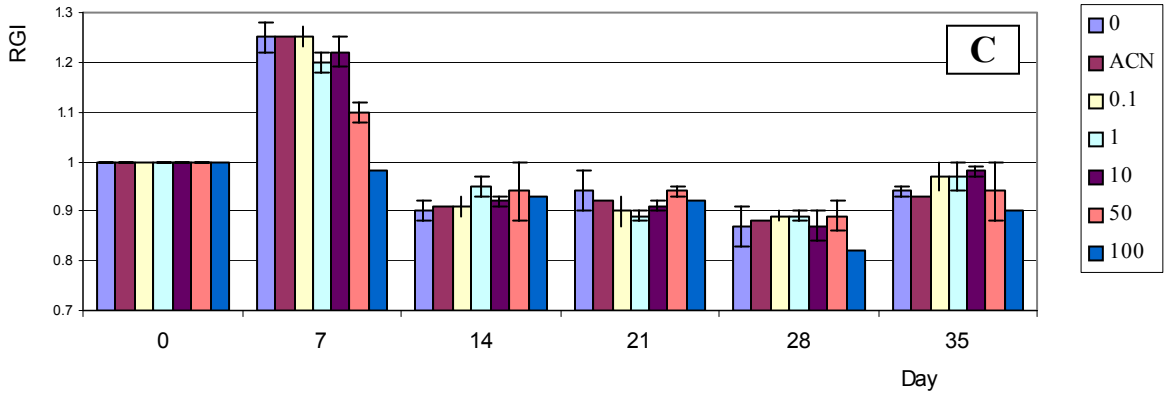
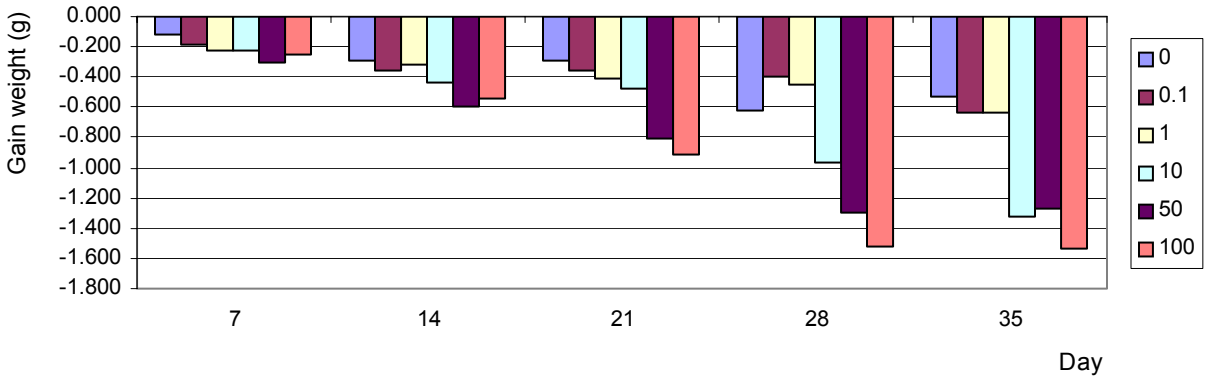
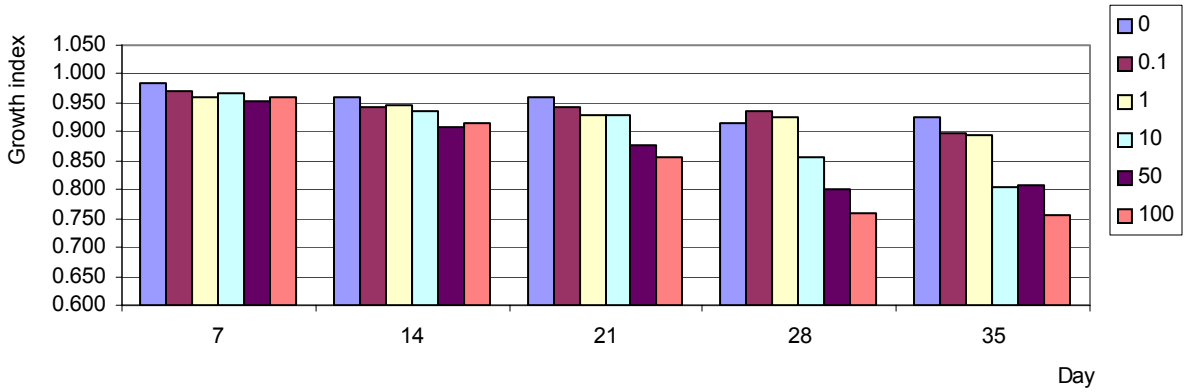


Figure 15.2.2. ***Effect of TNX on earthworm growth in amended sandy loam soil after 0, 7, 14, 21, 28 and 35 d of exposure (n = 10 earthworms X 3 replicates /***

concentration at start of experiments). Error bars show standard deviation. A. Weight gain. B. Growth Index (earthworm weight at certain time after exposed to amended soil related to original weight). C. Relative Growth Index (earthworm weight at certain time after exposed to amended soil related to the weight 7 days earlier).





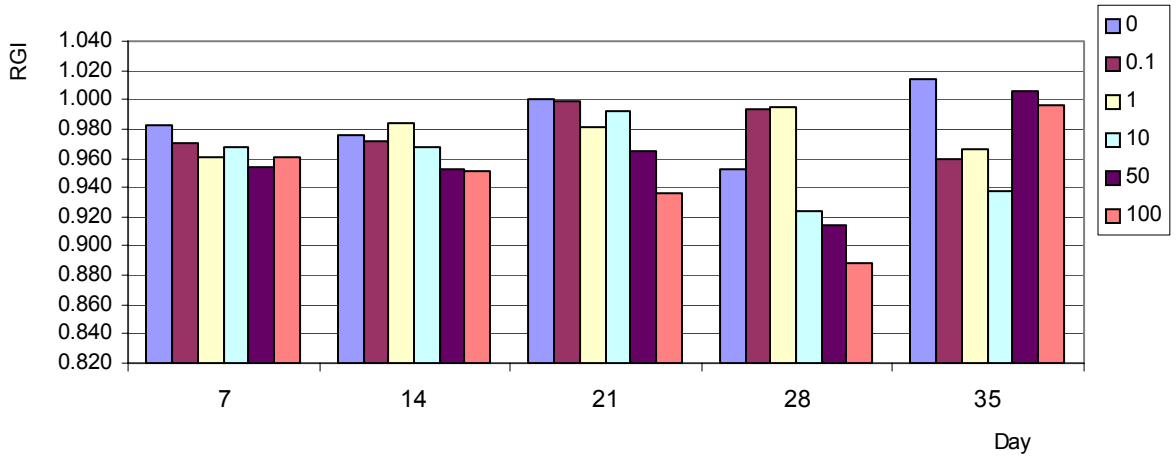


Figure 15.2.3. ***Effect of MNX on earthworms in amended sandy loam soil after 0, 7, 14, 21, 28 and 35 d of exposure (n = 10 earthworms X 3 replicates***

/ concentration at start of experiments). A. Weight gain. B. Growth Index (earthworm weight at certain time after exposed to amended soil related to original weight). C. Relative Growth Index (earthworm weight at certain time after exposed to amended soil related to the weight 7 days earlier).

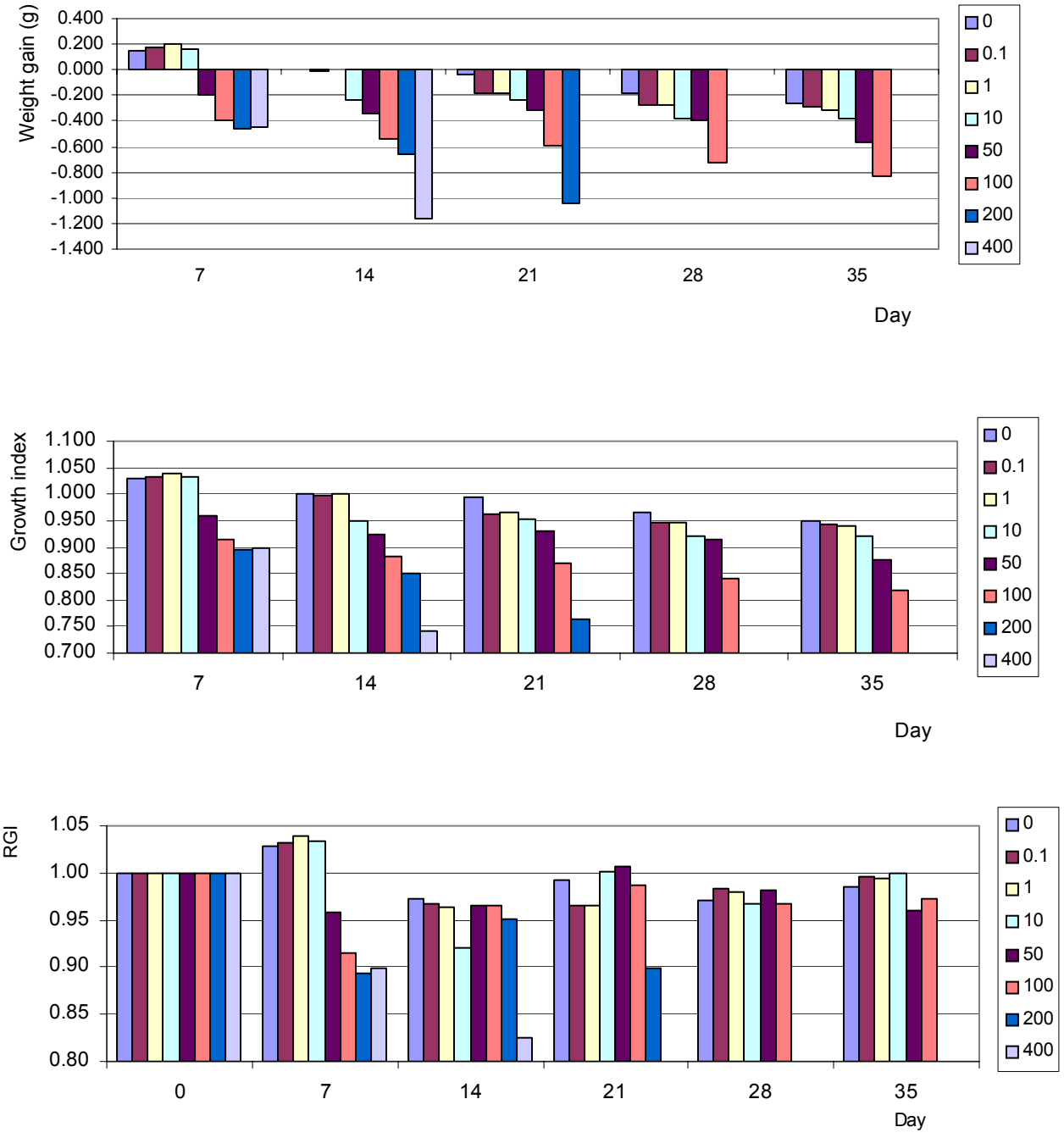
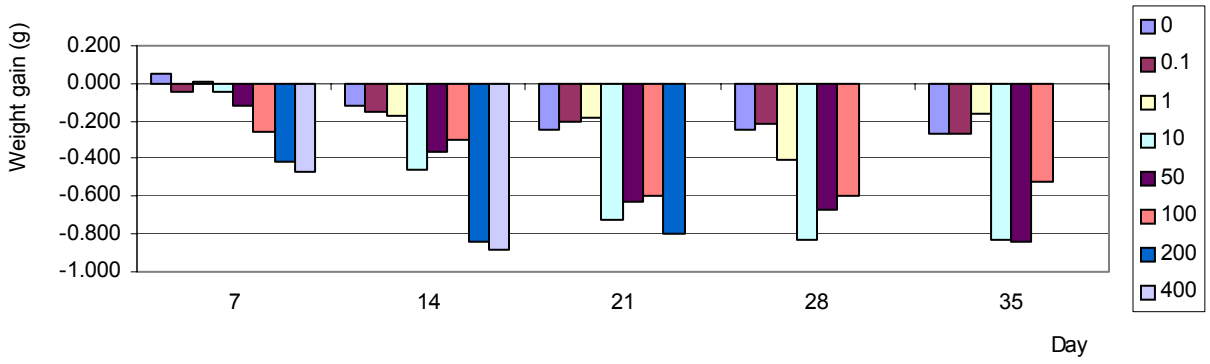
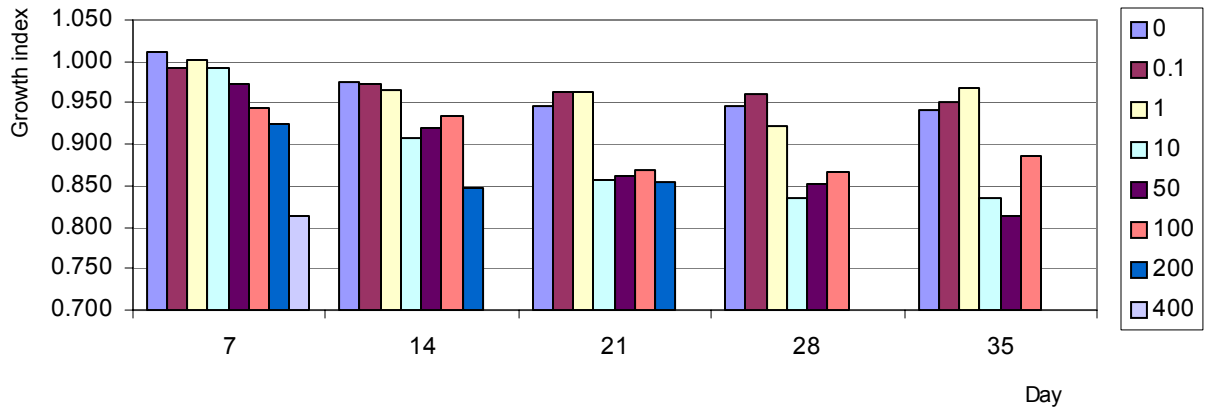


Figure 15.2.4. ***Effect of TNX on earthworms in amended silt loam soil after 0, 7, 14, 21, 28 and 35 d of exposure (n = 10 earthworms X 3 replicates / concentration at start of experiments). A. Weight gain. B. Growth Index (earthworm weight at certain time after exposed to amended soil related to original weight). C. Relative Growth Index (earthworm weight at certain time after exposed to amended soil related to the weight 7 days earlier).***





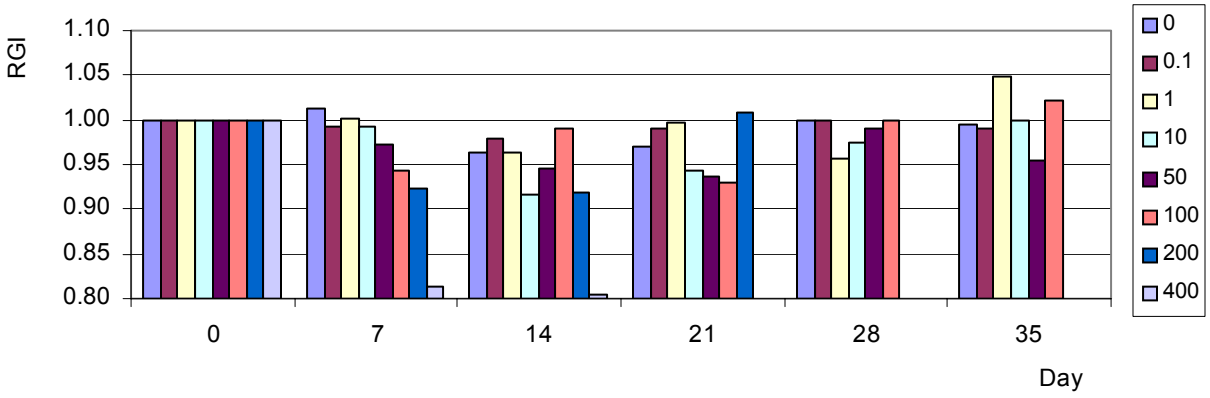
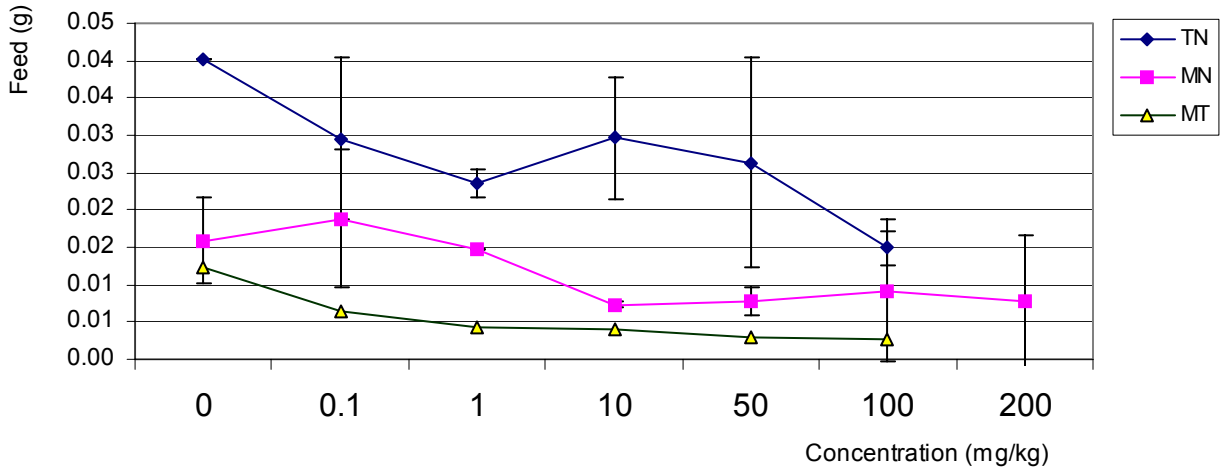


Figure 15.2.5. ***Effect of MNX on earthworms in amended silt loam soil after 0, 7, 14, 21, 28 and 35 d of exposure (n = 10 earthworms X 3 replicates / concentration at start of experiments). A. Weight gain. B. Growth Index (earthworm weight at certain time after exposed to amended soil related to***

original weight). C. Relative Growth Index (earthworm weight at certain time after exposed to amended soil related to the weight 7 days earlier).

A.



B.

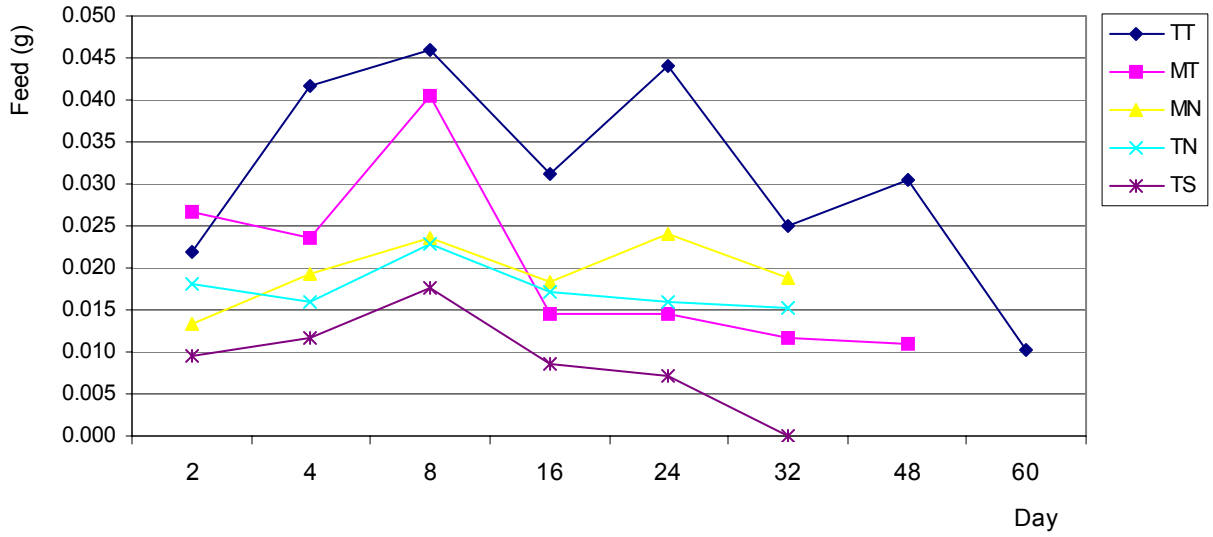


Figure 15.2.6. Effect of MNX and TNX on earthworm feeding in two types of amended natural soils and sand after exposure (n = 10 earthworms X 3 replicates / concentration at start of experiments). A. Effect of TNX or MNX concentration on earthworm feeding. C. Dynamic changes in feeding of earthworms with time.

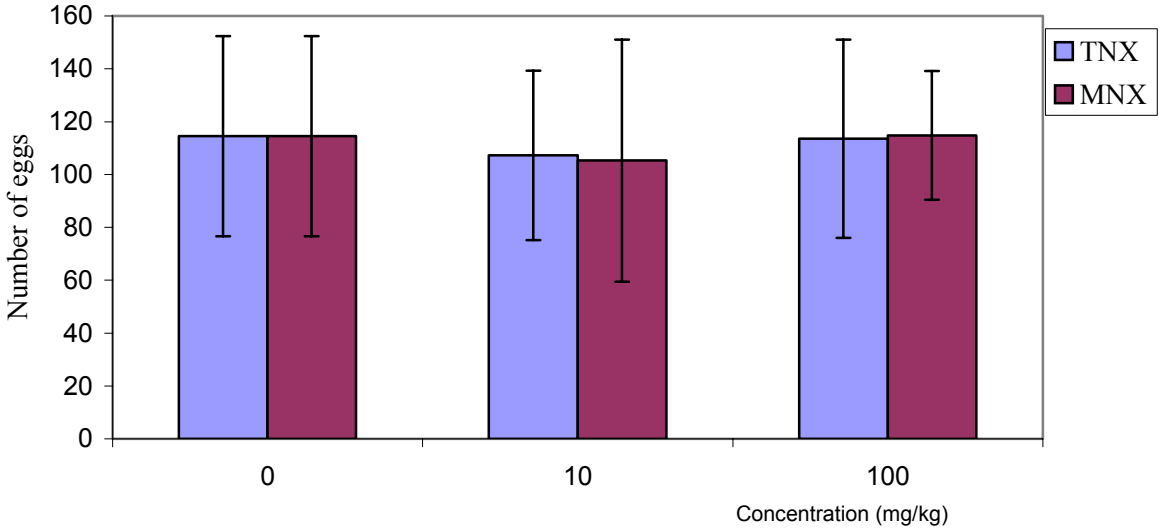


Figure 15.3.1 Effect of TNX or MNX feed on cricket egg production.

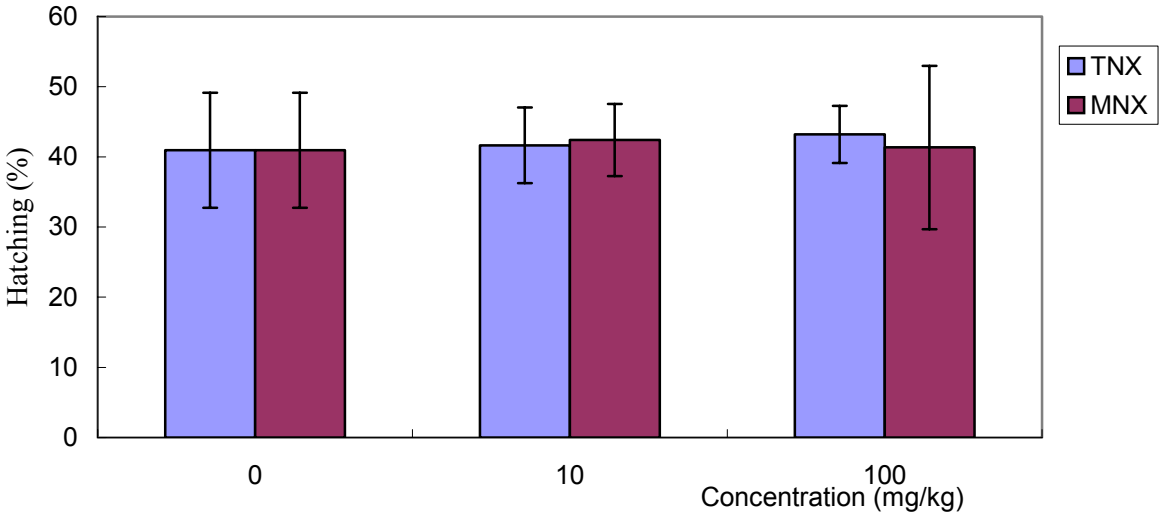


Figure 15.3.2 Effect of TNX- or MNX-fed crickets on egg hatching.

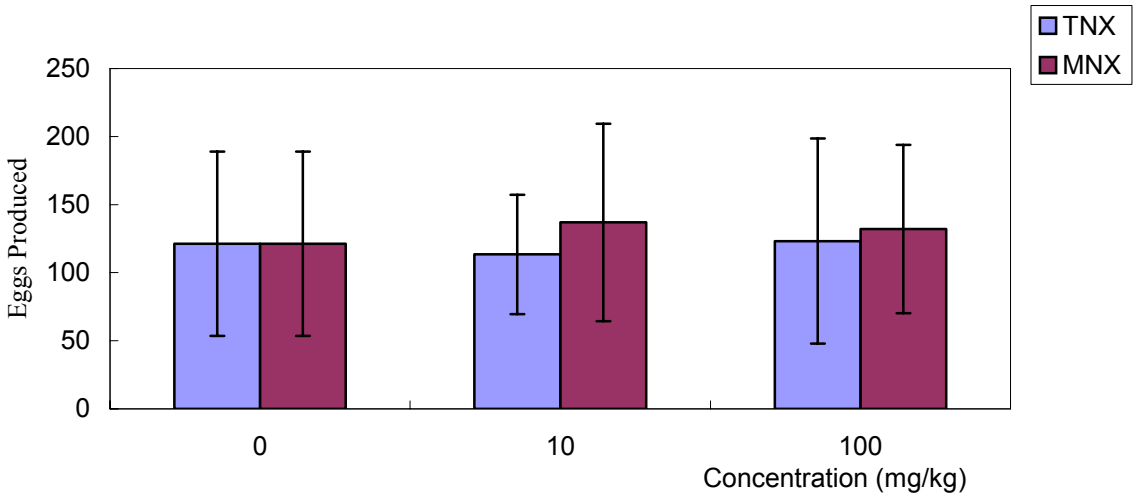


Figure 15.3.3 Eggs laid on TNX- or MNX-contaminanted sand.

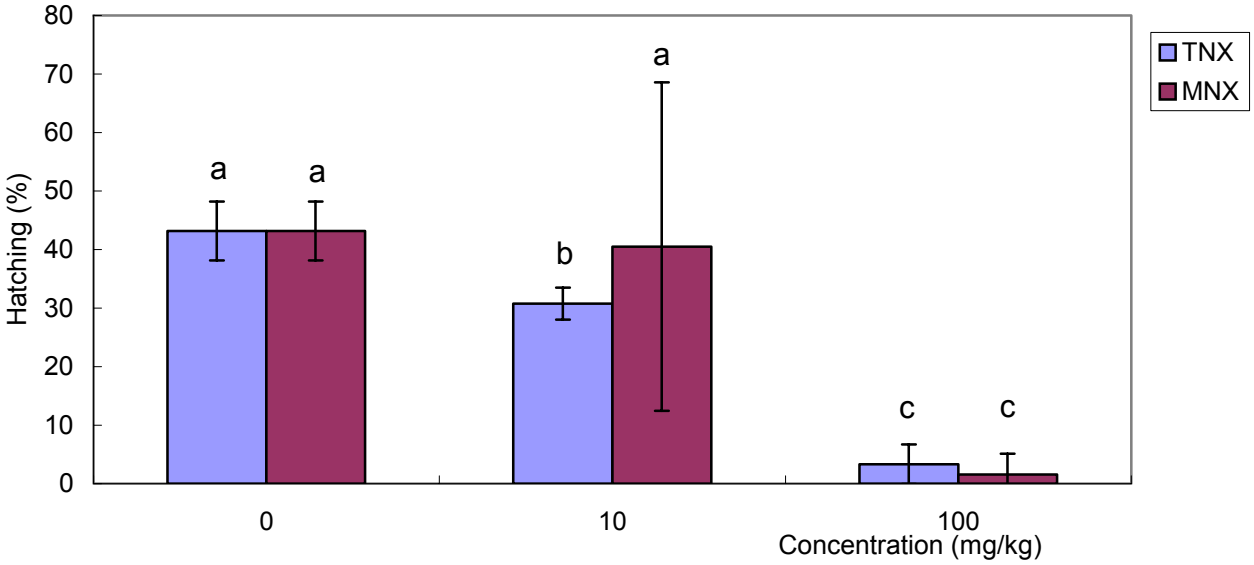


Figure 15.3.4 Effect of MNX or TNX on cricket egg hatching in contaminated sand.

Table 16.2.1. Lethal and sublethal ecotoxicological parameters for MNX and TNX determined in two types of amended natural soils (mg/kg).

		Regression	NOEC	LOEC	EC20 or LC20	EC50 or LC50
Sandy loam soil						
Survival (7 d)	MNX	Y=103.202-0.2030X (r2=0.987)	102.75	>102.75	114.30	262.08
	TNX	Y=104.378-0.2144X (r2=0.968)	43.90	97.14	113.70	253.63
Survival (14 d)	MNX	Y=103.202-0.2030X (r2=0.987)	102.75	>102.75	114.30	262.08
	TNX	Y=103.906-0.2145X (r2=0.978)	43.90	97.14	111.45	251.31
Survival (35 d)	TNX	Y=103.906-0.2145X (r2=0.978)	43.90	97.14	111.45	251.31
Growth (7 d)	MNX	ND	102.75	>102.75	ND	ND
	TNX	Y=1.2333-0.0026X (r2=0.998)	10.07	43.90	89.73	233.96
Growth (35 d)	MNX	Y=0.8984-0.0019X (r2=0.985)	0.741	9.124	82.53	228.89
	TNX	Y=0.9116-0.0019X (r2=0.996)	1.29	10.07	92.42	237.68
Silt loam soil						
Survival (7 d)	MNX	Y=124.936-0.1922X (r2=0.895)	211.67	>211.67	233.80	389.89
	TNX	Y=102.907-0.1145X (r2=0.937)	87.87	219.93	200.06	362.07
Survival (14 d)	MNX	Y=105.616-0.2274X (r2=0.938)	94.427	211.671	112.65	244.57
	TNX	Y=104.253-0.2508X (r2=0.930)	87.87	219.93	96.70	216.32
Growth (7 d)	MNX	Y=0.9999-0.0005X (r2=0.969)	45.80	94.43	399.80	999.80
	TNX	Y=0.9434-0.0014X (r2=0.994)	10.77	49.89	173.86	316.71
Growth (35 d)	MNX	Y=0.9206-0.0008X (r2=0.239)	1.17	12.96	150.75	525.75
	TNX	Y=1.0101-0.0014X (r2=0.657)	10.77	49.89	150.07	364.36

ND, not determined. ECs values could not determined because earthworm growth was not significantly ($p > 0.05$) different in treatment concentrations compared with carrier control.

Table 16.3.1. Toxicity comparison of RDX metabolites (MNX and TNX) based on sand tests (30 days of exposure) (μg/g)

RDX metabolite	EC	Sand test
MNX	20	21
	50	52
	95	99
TNX	20	12
	50	48
	95	97